Biochimica et Biophysica Acta, 732 (1983) 154–159
Elsevier

BBA 71730

ANALYSIS AND PURIFICATION OF THE BLOOD-SINUSOIDAL DOMAIN OF RAT LIVER PLASMA MEMBRANE

JANET A. TAYLOR [a], DURWARD LAWSON [b] and JACOB D. JUDAH [a]

Department of Experimental Pathology, The School and Medicine [a] and Department of Zoology [b], University College London, Gower Street, London WC1E 6JJ (U.K.)

(Received August 5th, 1982)
(Revised manuscript received March 21st, 1983)

Key words: Membrane domain; Blood-sinusoidal membrane; Ficoll gradient centrifugation; (Rat liver hepatocyte)

Methods are described for the analysis and purification of the blood-sinusoidal domains of rat liver plasma membranes using a combination of sucrose and Ficoll density gradient centrifugation. Use has been made of ^{125}I-labelled wheat-germ agglutinin and hormone-stimulated adenylate cyclase to identify the blood sinusoidal fraction, which may be resolved from Golgi and endoplasmic reticulum markers on Ficoll gradients.

Introduction

The plasma membrane of the hepatocyte is complex. It consists of three readily distinguishable domains, namely the blood-sinusoidal, the contiguous (that which apposes to neighbouring cells) and the bile canalicular. These three regions may be separated from each other by a combination of differential and gradient centrifugation after homogenisation in 250 mM sucrose. The blood-sinusoidal face sediments with microsomes, as shown by binding of ^{125}I-labelled wheat-germ agglutinin [1] after intraportal injection in situ, before homogenisation. This microsomal fraction has been separated and attempts have been made to purify it [2,3]. Its enzyme marker is hormone-stimulated adenylate cyclase. Whilst 5′-nucleotidase and alkaline phosphodiesterase are represented on the blood-sinusoidal face, they are enriched to a far greater extent in the contiguous and bile canalicular domains [3,4]. These regions of the plasma membrane are recovered in the so-called nuclear sediment by low speed centrifugation from sucrose homogenates and may be purified and separated from each other by density gradient centrifugation [2–4].

In this work, we are concerned with the blood-sinusoidal face of the rat hepatocyte. Its purification is fraught with problems and both the methods available [2,3] yield fractions which are apparently heavily contaminated. Attempts to purify these fractions on linear sucrose gradients were unsuccessful but centrifugation on linear Ficoll gradients allowed the separation from them of endoplasmic reticulum and Golgi elements. We report here the results of our analysis and a preparation of the blood-sinusoidal plasma membrane which is at least twice as pure as the starting material obtained from sucrose gradients.

Materials and Methods

Animals

Female Wistar rats (about 150 g body wt.) were obtained from A. Tuck & Sons, Battlesbridge, Essex, U.K.

Microsomal plasma membranes were prepared by a slight modification of the method of Aronson and Touster [5]. Only 10 g rat liver were processed at any time (instead of 20 g). This enabled us to reduce the volumes of the sucrose step-gradient used for the fractionation of the microsomal pellet

and so to use four tubes of the SW41 Ti rotor (Beckman Instruments Ltd, Glenrothes, Scotland) instead of the SW25.2 rotor. The gradients could then be centrifuged at $200000 \times g_{av}$ for 90 min instead of the overnight run specified in the original method.

The gradient in the SW41 Ti tube was composed of the load, 5 ml 49% (w/w) with respect to sucrose, 5 ml 34% (w/w) sucrose and 2.5 ml of 250 mM sucrose. For some experiments (as described in the Results section), the gradient was modified: above the load were introduced 3 ml 34% (w/w) sucrose then 2.5 ml 26% (w/w) sucrose and finally 2 ml of 250 mM sucrose. All the sucrose solutions contained 1 mM EGTA and 5 mM Tris-HCl (pH 8.0 at 20°C) as defined by Aronson and Touster [5]. The plasma membrane fraction floating on 34% (w/w) sucrose was removed, pelleted and suspended in 1–2 ml 70 mM sucrose/1.0 mM EGTA/5 mM Tris-HCl (pH 7.7). This preparation was then subjected to fractionation on linear Ficoll gradients as described below.

Fractionation of membranes on Ficoll gradients

All Ficoll solutions contained 5 mM Tris-HCl and 1.0 mM EGTA (pH 7.7 measured at 20°C). Linear gradients were generated at 2°C by using three channels of a peristaltic pump so as to make 12 ml of gradient (5–15.5% (w/w) Ficoll) in centrifuge tubes of the SW41 Ti rotor. A plasma membrane suspension (3–12 mg protein) was layered on the gradients, which were centrifuged for 16 h (Beckman L2 65B centrifuge) at $200000 \times g_{av}$. At the end of the run, the entire gradient was pumped out for marker enzyme assay.

For preparative purposes, discontinuous Ficoll gradients were used. Into SW41 Ti tubes were placed 1 ml 18% (w/w) Ficoll, overlayed with 5.5 ml 12% (w/w) Ficoll and 5.5 ml 8% (w/w) Ficoll. All the Ficoll solutions contained 5 mM Tris-HCl and 1.0 mM EGTA (pH 7.7 measured at 20°C). Above the gradient was layered a plasma membrane suspension, as described above. Centrifugation for 16 h at $200000 \times g_{av}$ separated a rather hazy layer floating on the 18% (w/w) Ficoll cushion from two sharp bands at the top of the 12% and the top of the 8% Ficoll layers, respectively. The plasma membrane band resided at the top of the 12% Ficoll layer. It was removed, diluted 5-fold with 250 mM sucrose/1.0 mM EGTA (pH 7.4) and the vesicles were sedimented ($100000 \times g_{av}$ for 60 min). The pellet was suspended in a convenient volume of 70 mM sucrose/1.0 mM EGTA (pH 7.4).

Enzyme assays

5′-Nucleotidase activity (EC 3.1.3.5) was determined by the method of Newby et al. [6]. Adenylate cyclase (EC 4.6.1.1), glucose-6-phosphatase (EC 3.1.3.9), NADPH-cytochrome *c* oxidoreductase (EC 1.6.1.4), succinate dehydrogenase (EC 1.3.99.1) and monoamine oxidase (EC 1.4.3.4) were assayed as described by Wisher and Evans [3]. Fluoride-stimulated adenylate cyclase activities were measured in the presence of 15 mM $MgCl_2$ and 10 mM NaF. Cyclic AMP was assayed with a commercial kit (Amersham International, Amersham Bucks., U.K.) UDPgalactose:ovalbumin-galactosyltransferase (EC 2.4.1.38) was assayed by the method of Hino et al. [7]. *β-N*-Acetyl-D-hexosaminidase (EC3.2.1.52) was assayed by the method of Barrett and Heath [8] using 4-methylumbelliferyl-2-acetamido-2-deoxy-*β*-D-glucopyranoside as substrate. Alkaline phosphodiesterase (EC 3.1.4.1) was determined by the method of Razzel [9]. The enzyme was inactivated by prolonged exposure to EGTA, which was therefore excluded from the relevant gradients.

Protein was measured by the method of Lowry et al. [10] using bovine serum albumin as a standard. Sialic acid content of the membrane fractions was done with neuraminidase in the presence of Triton X-100 0.2% (v/v) [11]. Sialic acid was determined by the method of Warren [12]. Labelling of the blood-sinusoidal domain of the hepatocyte plasma membrane was done in situ with wheat-germ agglutinin labelled with ^{125}I to a specific radioactivity of $8 \cdot 10^6$ cpm/μg protein and injected into the liver as previously described [1] in a dose of about 2 μg. The lectin was administered in 10 ml ice-cold Krebs-Ringer solution (pH 7.3) containing 1% (w/v) bovine serum albumin. After 5 min (during which the liver was irrigated with ice-cold saline), a further 20 ml of ice-cold Krebs-Ringer solution was perfused through the liver, and then 20 ml of 250 mM sucrose.

Density frequency histograms, used to show the

distribution of marker enzymes following density gradient centrifugation were constructed as described by Beaufay et al. [13].

Electron microscopy

Fractions were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 30 min at room temperature, washed in lysine (25 mg/ml) in phosphate buffer and then in buffer alone. Samples were then labelled with 200 μl of wheat-germ agglutinin or concanavalin A coupled to ferritin for 30 min at room temperature, washed six times in phosphate buffer to remove unbound conjugate and then postfixed in 1% OsO_4 in phosphate for 1 h at 4°C, dehydrated and embedded in Epon 812 [14].

At least 20 separate fields from each experiment were photographed and a total of approx. 300 vesicles were counted to give the data shown. Random sampling was performed by cutting the pellet at various levels.

Ferritin conjugates

Lectins were conjugated to ferritin by glutaraldehyde, purified on sucrose density gradients using the method of De Petris and Raff [15] and used at a final concentration of 500 μg of lectin/ml.

Cytochrome *c* (type 6), NADPH, glucose 6-phosphate (Na salt), AMP, ATP, UDPgalactose, ovalbumin and neuraminidase type 9 were obtained from Sigma Chemical Co., Poole, Dorset, U.K. UDP[^{14}C]galactose and [^{3}H]AMP were obtained from Amersham International, U.K. Ficoll 400, and wheat-germ agglutinin were obtained from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, U.K. Bovine serum albumin, A grade, was obtained from Calbiochem-Behring Corp., C.P. Laboratories, Bishops Stortford, Herts., U.K.

Results and Discussion

Analysis of plasma membrane fraction on linear Ficoll gradients

Fig. 1 shows the excellent separation of the various components of the plasma membrane fraction obtained from the sucrose gradient. It is plain that Golgi and endoplasmic reticulum markers are well separated from the plasma membrane markers. It should be noted that glucagon-stimulated adenylate cyclase co-migrates with the ^{125}I-labelled-wheat-germ agglutinin, suggesting very strongly that these fractions are derived from the blood-sinusoidal domain. It can also be seen that these two markers do not co-migrate with 5′-nucleotidase and alkaline phosphodiesterase. There are ready explanations for this. One is that 5′-nucleotidase and alkaline phosphodiesterase have a considerable representation in Golgi membranes [18]. The second is that the microsomal plasma membrane fraction is contaminated with fragments from the bile canalicular face of the hepatocyte, where both enzymes have a marked concentration [3]. A third possibility for the 5′-nucleotidase at least arises from an unidentified intracellular pool of this enzyme [19].

Effect of sucrose on Ficoll gradients

We made up the Ficoll solutions for the gradient in 250 mM sucrose to see if this would improve resolution. On the contrary, there was no resolution on the gradient which resembled the linear sucrose gradients tested unsuccessfully [3] and in the present work.

Method for preparation of purified plasma membranes

Following the analysis of the plasma membrane fraction on linear Ficoll gradients, we decided to devise a preparative procedure using step gradient of Ficoll. Since the membranes were heavily contaminated with Golgi elements, we attempted to remove these before placing them on the Ficoll gradients. To this end, we modified the Aronson and Touster sucrose gradient described in the Materials and Methods section by introducing a layer of 26% (w/w) sucrose above the 34% (w/w) sucrose layer. Centrifugation of this gradient at $200\,000 \times g_{av}$ for 90 min resulted in a band floating on the 26% sucrose layer. Marker analysis showed that this band had the properties of a purified Golgi preparation [7,16,17]. It contained 25% of the protein of the original plasma membrane preparation, with 50% of the galactosyltransferase activity, which was 120-fold enriched over the original homogenate. There were low levels of plasma membrane markers. It was therefore discarded, and the plasma membrane

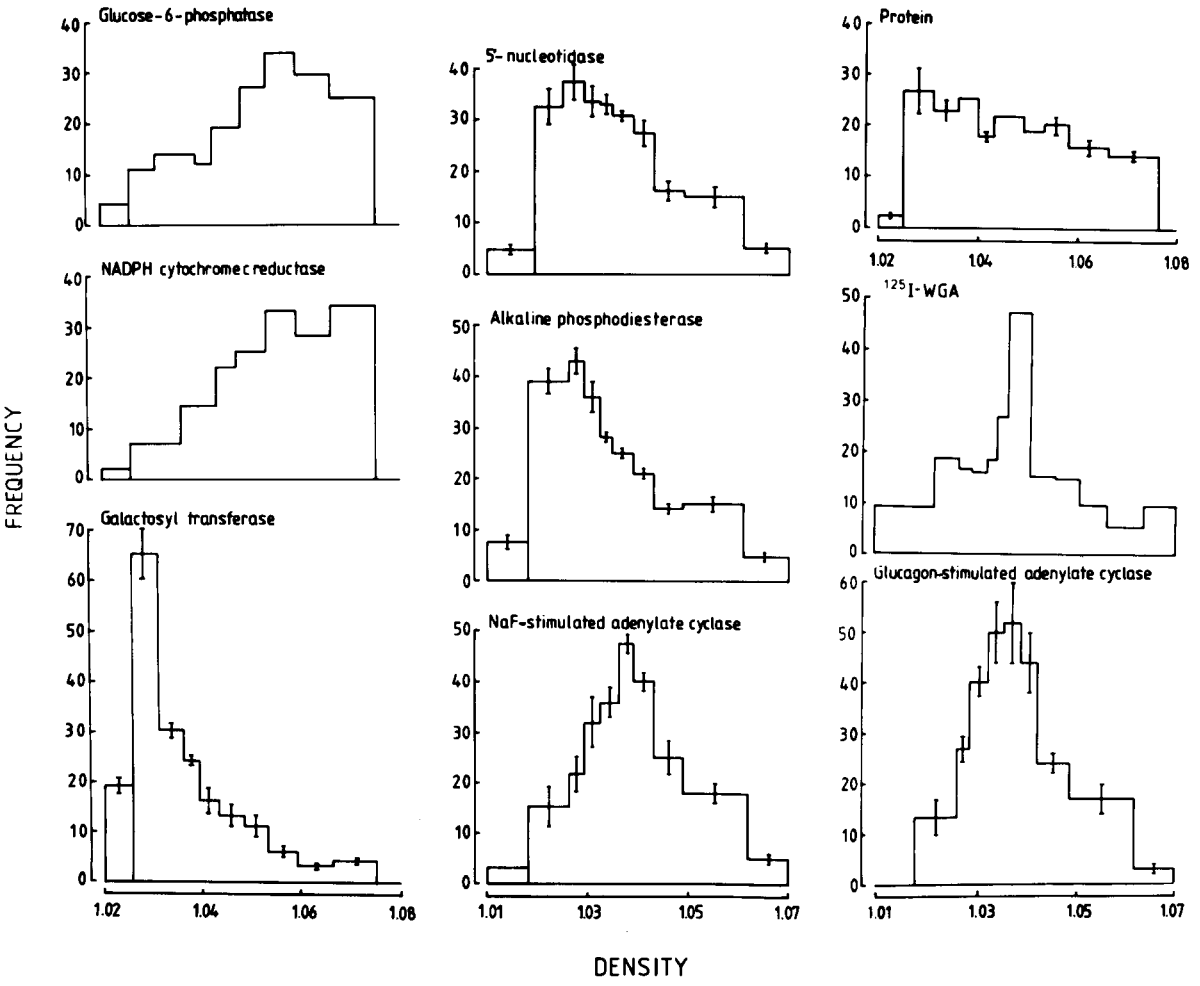

Fig. 1. Resolution of enzyme markers on linear Ficoll gradients. 1-ml fractions were collected from the gradients. Ficoll concentrations were determined by refractometry. Six runs were done, but glucose-6-phosphatase, NADPH-cytochrome *c* oxidoreductase and labelling with ^{125}I-labelled wheat-germ agglutinin (^{125}I-WGA) were measured three times. The bars represent S.E.

fraction floating on the 34% sucrose was collected and used for further purification on the step gradients of Ficoll which are described in the Materials and Methods section. Table I shows the results. Approx. 70% of the glucagon-stimulated cyclase was recovered with a doubling of its specific activity, the enrichment of the sialic acid, which is concentrated on plasma membranes [20], doubled, approx. 70% of the protein, glucose-6-phosphatase, NADPH-cytochrome *c* oxidoreductase, galactosyltransferase and alkaline phosphodiesterase were removed.

Electron microscopy

The blood-sinusoidal face of the hepatocyte forms vesicles upon homogenisation (see Ref. 4 for a discussion). Electron micrographs of the sucrose fraction showed a picture very similar to those published by Wisher and Evans [3], the field consisting of vesicles, together with structures identifiable as Golgi cisternae. We examined the fraction separated on step gradients of Ficoll by fixation in glutaraldehyde and subsequent labelling with wheat-germ agglutinin or with concanavalin A each conjugated with ferritin. Low-power micrographs

TABLE I

ACTIVITIES OF ENZYME MARKERS IN PLASMA MEMBRANE FRACTIONS

Specific activities (mean ± S.E.) from six determinations are expressed as μmol product formed per h per mg protein, except for galactosyltransferase, NADPH-cytochrome *c* oxidoreductase, succinate dehydrogenase and adenylate cyclase which are expressed as nmol product formed per h per mg protein. Protein is expressed in mg and sialic acid as nmol per mg protein. Enrichment of enzyme activity is related to the mean specific activities of at least five homogenates. The enrichment of sialic acid is related to its content (nmol/mg protein) in the original homogenates. The preparations were as described in Materials and Methods and in the text. n.d., none detected.

Enzyme	Original plasma membrane fraction			After Ficoll gradient		
	Specific activity	Enrich-ment	Yield (% of homogenate)	Specific activity	Enrich-ment	Yield (% of homogenate)
Glucose-6-phosphatase	13.4 ± 1.3	1.4	1.6	13.0 ± 2.22	1.4	0.4
NADPH-cytochrome *c* oxidoreductase	1.0 ± 0.18	2.6	3.0	0.92 ± 0.12	2.4	0.7
Succinate dehydrogenase	9.0 ± 1.1	0.008	0.01	1.2	0.001	0.003
Monoamine oxidase	0.29 ± 0.03	3.2	3.7	n.d.	n.d.	n.d.
Hexosaminidase	0.48 ± 0.03	0.5	0.6	0.53 ± 0.05	0.5	0.15
Galactosyltransferase	320 ± 32	55	64	148 ± 15	26	7.8
5′-Nucleotidase	17.4 ± 2.0	15	18	27.3 ± 2.2	23	6.9
Alkaline phospho-diesterase	33.8 ± 8.3	21	25	37 ± 2.1	23	6.9
Basal adenylate cyclase	0.42 ± 0.07	4.8	5.6	1.03 ± 0.13	11.7	3.5
F^--stimulated adenylate cyclase	3.68 ± 0.37	24.5	29	8.72 ± 1.7	58	17
Glucagon-stimulated adenylate cyclase	2.30 ± 0.23	10.0	12	6.2 ± 0.8	27	8.1
Sialic acid	28.8 ± 3.0	8.0	9.4	63.6 ± 4.0	18	5.4
Protein	21.0 ± 0.9	–	1.2	5.2 ± 0.3	–	0.3

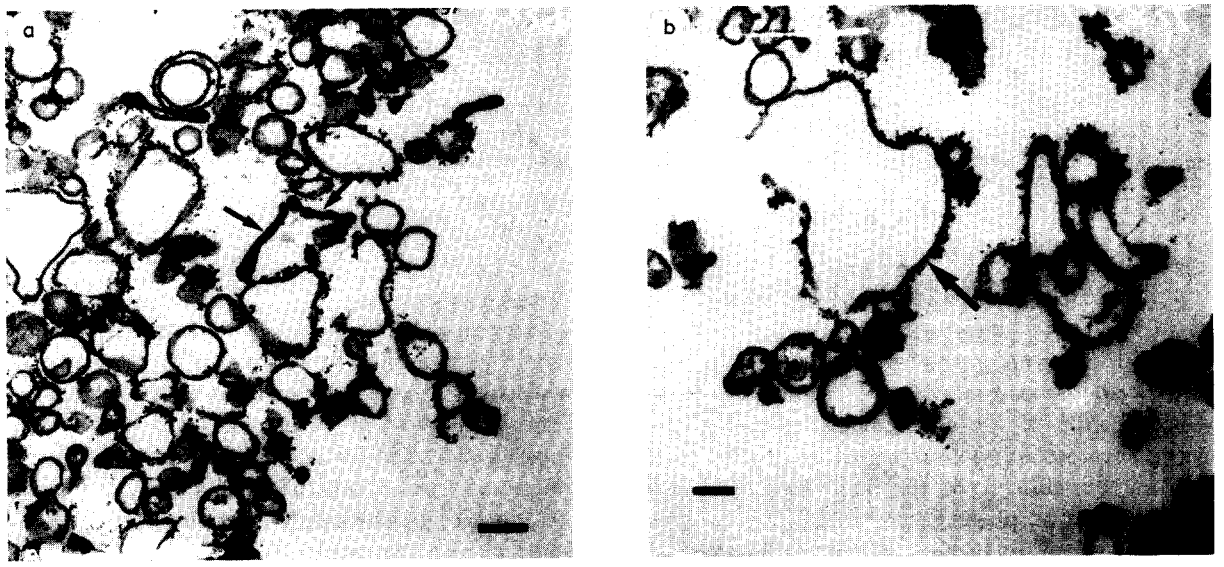

Fig. 2. Electron micrograph of plasma membrane vesicles after treatment with wheat-germ agglutinin-ferritin conjugate. The vesicles were separated by centrifugation on a discontinuous Ficoll gradient and electron microscopy was done as described in the Materials and Methods section. (a) The attachment of the ligand to the outside of many of the vesicles. The arrow indicates what may be two collapsed Golgi cisternae elements. (b) The arrow shows a broken vesicle, with wheat-germ agglutinin-ferritin disposed on one side only. The bar represents 1 μm.

of the pellets cut at different levels showed reasonable homogenity, with negligible mitochondrial contamination, and some Golgi cisternae. A vesicle count revealed that 50%–60% of all vesicles present were specifically labelled with ferritin-conjugated lectins, using the criteria for specificity of De Petris and Raff [15]. Approx. 15% of the vesicles were recognisable Golgi cisternae. Samples incubated with free competing sugars (100 mM *N*-acetylglucosamine or 100 mM α-methylmannoside) showed no specific labelling. We may conclude, therefore, that at least 50% of the vesicles present are derived from plasma membranes. However, this is a minimum figure, since some of the vesicles may be everted, that is inside-out, in which case they would not be labelled by the lectin. Fig. 2 shows a representative field from a preparation treated with ferritin wheat-germ agglutinin.

Conclusions

From the enrichment in glucagon-stimulated adenylate cyclase and in sialic acid, it can be concluded that the plasma membrane fraction recovered from the Ficoll gradients has been doubled in purity as compared to the original (Table I). Since the ^{125}I-labelled wheat-germ agglutinin co-migrates with the cyclase (see Fig. 1), it can also be concluded that the plasma membrane fraction is derived from the blood-sinusoidal domain. The use of this second marker confirms the belief that hormone-stimulated adenylate cyclase is indeed an excellent marker for the blood-sinusoidal face of the hepatocyte, as suggested previously [3,4], particularly as only about 2 μg of wheat-germ agglutinin were injected into the liver intravascularly and in situ so as to minimise nonspecific binding [1]. There is little enrichment of 5′-nucleotidase, but this is not surprising in view of the fact that this marker did not co-migrate with the ^{125}I-labelled wheat-germ agglutinin or with adenylate cyclase, and is in accord with the conclusion [3] that the greatest enrichment of 5′-nucleotidase is to be seen in the bile canalicular and contiguous faces of the rat-hepatocyte membranes. The presence of endoplasmic reticulum markers suggests some contamination by microsomes, but one must be aware that these enzymes are not located uniquely in any one subcellular component [18,21,22].

We believe that our preparations are an improvement on those hitherto published [2,3]. The latter are a good deal more contaminated than may be realised (see Ref. 2, where it is suggested that the microsomal fraction of plasma membranes may be 80% pure). We should also like to draw attention to the use of Ficoll as a medium for density-gradient centrifugation under circumstances where sucrose may not be satisfactory.

Acknowledgements

We are indebted to the Vandervell Foundation, the Medical Research Council and the Cancer Research Campaign for grants.

References

1 Chang, K.J., Bennett, V. and Cuatrecasas, P. (1975) J. Biol. Chem. 250, 488–500
2 Touster, O., Aronson, N.N., Dulaney, J.T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–618
3 Wisher, M.H. and Evans, W.H. (1975) Biochem. J. 146, 375–388
4 Evans, W.H. (1980) Biochim. Biophys. Acta 604, 27–64
5 Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31, 90–102
6 Newby, A.C., Luzio, J.P. and Hales, C.N. (1975) Biochem. J. 146, 625–633
7 Hino, Y., Asano, A., Sato, R. and Shimizu, S. (1978) J. Biochem. 83, 909–923
8 Barrett, A.J. and Heath, M.F. (1977) in Lysosomes: A Laboratory Handbook, 2nd Edn. (Dingle, J.T., ed.), pp. 19–145, Elsevier/North-Holland, Amsterdam
9 Razzel, W.E. (1963) Methods Enzymol. 6, 236–258
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
11 Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 2, pp. 245–282, Plenum Press, New York
12 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
13 Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O.Z., Berthet, J. and De Duve, C. (1964) Biochem. J. 92, 184–205
14 Lawson, D., Raff, M.C., Gomperts, B.D., Fewtrell, C. and Gilula, B. (1977) J. Cell Biol. 72, 242–259
15 De Petris, S. and Raff, M.C. (1972) Eur. J. Immunol. 2, 523–535
16 Ehrenreich, J.H., Bergeron, J.J.M., Siekevitz, P. and Palade, G.E. (1973) J. Cell Biol. 59, 45–72
17 Bergeron, J.J.M. (1979) Biochim. Biophys. Acta 555, 493–503
18 Howell, K.E. and Palade, G.E. (1982) J. Cell Biol. 92, 822–832
19 Stanley, K.K., Edwards, M.R. and Luzio, J.P. (1980) Biochem. J. 186, 59–69
20 Amar-Costesec, A. (1981) J. Cell Biol. 89, 62–69
21 Howell, K.E., Ito, A. and Palade, G.E. (1978) J. Cell Biol. 79, 581–589
22 Ito, A. and Palade, G.E. (1978) J. Cell Biol. 79, 590–597