Biochimica et Biophysica Acta, 642 (1981) 313—324

BBA 79170

THE PURIFICATION OF PLASMA MEMBRANES FROM WI-38 FIBROBLASTS

EFFECTS OF AGEING ON THEIR COMPOSITION

MARTINE RAES, ANDRÉE HOUBION and JOSÉ REMACLE *

Unité de Biochimie Cellulaire, Facultés Universitaires de Namur, Rue de Bruxelles 61, B-5000 Namur (Belgium)

(Received September 25th, 1980)

Key words: Plasma membrane purification; Aging; Membrane composition; (Human fibroblast)

Summary

A three-step method for the purification of plasma membranes from WI-38 fibroblasts was developed thus allowing the recovery of 36—44% of the plasma membrane. Except in the case of galactosyltransferase, the activity of the contaminating enzymes was very low. Morphological observations confirm the presence of a homogeneous population of vesicles.

Preparations obtained from young and old cell cultures were compared for their enzymatic and protein contents. With ageing the activity of 5'-nucleotidase significantly increases whereas that of alkaline phosphodiesterase I decreases. Out of the 26 components detected after sodium dodecyl sulphate polyacrylamide gel electrophoresis, four decreased but only one increased. Cellular ageing seems to fulfil a specific and localized effect on the plasma membrane.

Introduction

The ageing of cells in cultures using human fibroblasts was described for the first time by Hayflick and Moorhead [1] and is now accepted as a general phenomenon affecting a great number of components and functions of eukaryotic cells [2,4]. Biochemical studies of cell components have put forward both quantitative (see reviews by Hayflick [5] and Cristofalo [2]) and

* To whom reprint requests should be addressed.
Abbreviation: SDS, sodium dodecyl sulphate.

qualitative [6,8] differences between young and old cells. Fewer membrane analyses were performed [9,11] and were mostly concerned with changes in the activities of membrane-bound enzymes [12,13]. On the other hand, studies on subcellular organelles have shown only slight differences in their functions [4] and physical parameters [14], whereas morphological observations have shown significant changes in their aspects [15,19]. Plasma membranes are particularly affected since on ageing, cells become thinner and larger, the number of dichotomized filopodia increases and numerous microvilli can be observed; moreover, in old cultures the cell density is low, thus suggesting that there is an increase in the density-dependent inhibition of growth.

Considering these indications and the possible role of the plasma membrane function in cellular ageing [20], we decided to perform a systematic study of plasma membrane modifications during the ageing of cells in culture. We first devised a method for the purification of membranes based on an analytical study of subcellular organelles [14]. The prepared membranes were then compared for their enzymatic and protein contents using SDS-polyacrylamide gel electrophoresis. The ageing of the cells was found to affect specifically five membrane components.

Materials and Methods

Cell culture. WI-38 fibroblasts were purchased from the American Type culture collection. Cells were subcultured according to the method of Hayflick [21]. Plasma membranes were prepared from two cell populations. (a) Young cells which grew exponentially and were subcultured 24—33 times (population-doubling level). These were in phase II of the culture described by Hayflick [21]. The Cristofalo index, i.e., the percentage of cells incorporating [^{3}H]thymidine in 24 h, was between 70 and 80. These high values are typical of a young culture as observed by Cristofalo and Sharf [22]. (b) Old cells which grew slower were subcultured 48—52 times and were in phase III of the culture. Their Cristofalo index of 40 was consistent with the age of the culture. The percentage of dead cells measured by the Trypan blue exclusion method was less than 1% in both cultures. The maximum population-doubling level reached by cells of the same lineage was 54—55. Cells were checked for contamination by mycoplasms but the results were negative.

Cell fractionation and effects of digitonin on the equilibration of subcellular organelles. Cells were harvested and fractionated as described in a preceding paper [14]. Unless otherwise stated, cells were harvested only from confluent roller bottles (8 days after subcultivation for young cells and 15 days for old cells). To test the effects of digitonin on subcellular organelles, we prepared an extract from young cells and added the equivalent of 0.12 mg digitonin per mg protein. The treated material was layered on a continuous sucrose gradient, centrifuged and handled as previously described [14].

Plasma membrane preparation. Plasma membranes were prepared from young and old cells in the following way. The cell homogenate prepared from six to eight roller bottles was fractionated by differential centrifugation in order to prepare a microsomal (P) fraction [14]; 2.3 ml of this fraction were layered in the tube of an SW 65 rotor already filled with 0.5 ml of 52.17%

(w/w) sucrose (density 1.25 g/cm^3), 2.0 ml of 33.48% (w/w) sucrose (density 1.15 g/cm^3) and 0.5 ml of 16.57% (w/w) sucrose (density 1.07 g/cm^3).

After centrifugation at 258 000 × *g* (r_{av} 6.4 cm) for 4 h at 4°C in an L5-65 Beckman ultracentrifuge, the membranes floating at the interface of density 1.07—1.15 g/cm^3 were collected (MP fraction) and diluted twice with a solution of digitonin which contained the equivalent of 0.3 mg digitonin per mg membrane protein.

A second gradient was prepared with 0.5 ml of 52.17% (w/w) sucrose (density 1.25 g/cm^3), 2.5 ml of 37.40% (w/w) sucrose (density 1.17 g/cm^3) and 2.2 ml of the MP fraction (density 1.08 g/cm^3), respectively. After equilibration by centrifugation at 179 000 × *g* (r_{av} 6.4 cm) for 16 h, the membranes at the interface of density 1.17—1.25 g/cm^3 were recovered and constituted the purified plasma membrane preparation (MP_1 fraction).

Technical analysis. Digitonin was a product of Merck AG, Darmstadt, F.R.G. The source of the other chemicals was the same as that used by Beaufay et al. [23].

Enzyme assays were described earlier [14].

Electron microscopy was carried out on samples prepared by the filtration of the membranes on Millipore filtres as described by Baudhuin et al. [24].

Electrophoresis on SDS-polyacrylamide gels was performed in a slab gel apparatus following the method of Laemmli [25] using the disc electrophoresis system described by Ornstein [26].

Results

Plasma membrane preparation

To develop a purification method, we took advantage of the analytical work [14] which showed that plasma membranes sediment mostly in the microsomal fraction after fractionation of the homogenate by differential centrifugation and also equilibrate in light fractions after isopycnic centrifugation in density gradients. The use of these two properties was not sufficient to allow a good separation of the plasma membrane from the other organelles so we examined the influence of a small amount of digitonin on the behaviour of the plasma membrane [27]. The results of the analytical gradient obtained with and without digitonin treatment of subcellular organelles are shown in Fig. 1. Alkaline phosphodiesterase (EC 3.1.4.1) and 5′-nucleotidase (EC 3.1.3.5) were used as enzyme markers of the plasma membrane [28]. Their median equilibrium density was found to increase on average from 1.135 to 1.184 g/cm^3 after digitonin treatment. The other subcellular organelles were hardly affected except galactosyltransferase (EC 2.4.1.38) which presented an increase in its median density equilibration of 0.008 g/cm^3.

The purification procedure described in Materials and Methods is a three-step method which takes advantage of the three above-mentioned properties. Biochemical analyses of the fractions obtained after each step are summarized in Table I. They represent the average of two preparations from young cells and two from old cells. During the purification procedure, the purity of the plasma membrane is indicated by the increasing values of the relative specific activity of the two plasma membrane enzymes starting from 2.8 in the micro-

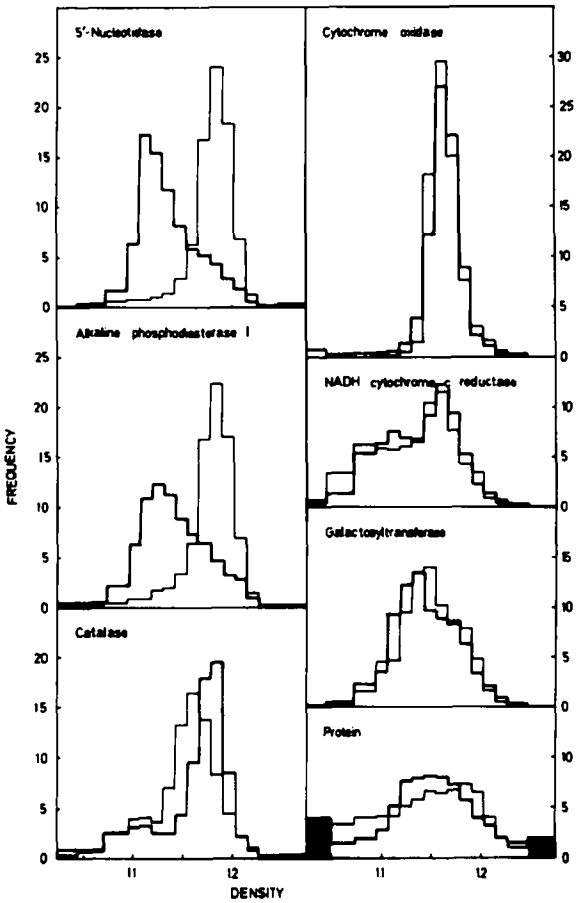

Fig. 1. Distribution patterns of enzymes after the isopycnic equilibration of an MLP fraction treated with (thin lines) or without (thick lines) digitonin. An extract from young cells was centrifuged for 30 min at 100 000 X *g* in order to prepare a complete particulate fraction (MLP) which was then equilibrated in a sucrose gradient as described in a previous paper [14]. For digitonin treatment, a complete particulate fraction (MLP) containing 8 mg protein was resuspended in 2.5 ml of 0.25 M sucrose containing 1.2 mg digitonin. The fraction was then analysed as before. The shaded areas on each side of the distribution profiles represent over arbitrary abscissa intervals, the enzymes recovered below a density of 1.045 and over 1.248 g/cm^3. Recoveries from the MLP fraction ranged between 82 and 121%.

somal fraction, then 5.2 and 4.9 in the MP fraction to reach 13 and 10 in the MP_1 fraction. On the other hand, the contaminating enzymes are gradually eliminated: cytochrome oxidase (EC 1.9.3.1) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), associated with mitochondria [29] and lysosomes [30], respectively, mostly disappear in the preparation of the microsomal fraction, particulate catalase (EC 1.11.1.6), associated with peroxisomes [31] in the first gradient, and NADH:cytochrome *c* reductase (EC 1.6.99.3), located in the endoplasmic reticulum and in the mitochondria [14] in the second gradient. Only a slight amount of galactosyltransferase is still recovered in the plasma membrane preparation. Compared to the homogenate, 63, 51 and 40%, respectively, of the plasma membrane enzymes are recovered in the three fractions, whereas in the MP_1 fraction other contaminating enzymes only represent

TABLE I

BIOCHEMICAL PROPERTIES OF PLASMA MEMBRANE PREPARATIONS

Values are given as means of four experiments ± S.D., and are corrected for the recovery which usually ranged between 90 and 130%. The relative specific activity is calculated with respect to the homogenate.

Constituent	Yield (% of homogenate)			Relative specific activity		
	P	MP	MP_1	P	MP	MP_1
Protein	22.42 ± 1.24	10.14 ± 0.34	3.67 ± 0.22			
Alkaline phosphodiesterase I	62.02 + 3.85	52.30 ± 8.18	44.37 ± 4.83	2.78 ± 0.27	5.16 ± 0.96	12.96 ± 0.10
5'-Nucleotidase	63.73 ± 2.79	50.65 ± 6.36	36.01 ± 6.78	2.79 ± 0.94	4.93 ± 0.19	9.87 ± 1.06
Cytochrome oxidase	12.89 ± 4.32	4.73 ± 0.79	0.54 ± 0.17	0.58 ± 0.22	0.47 ± 0.08	0.15 ± 0.06
NADH:cytochrome *c* reductase	47.57 ± 7.17	27.20 ± 4.51	0.49 ± 0.22	2.14 ± 0.43	2.69 ± 0.53	0.14 ± 0.06
Galactosyltransferase	68.41 ± 2.81	39.51 ± 5.08	7.95 ± 0.54	3.20 ± 0.16	3.98 ± 0.57	2.22 ± 0.01
N-Acetyl-D-glucosaminidase	19.31 ± 3.11	6.63 ± 3.15	2.87 ± 1.51	0.86 ± 0.18	0.66 ± 0.32	0.77 ± 0.42
Catalase	41.88 ± 5.84	7.48 ± 2.51	1.81 ± 0.14	1.88 ± 0.33	0.75 ± 0.27	0.49 ± 0.07

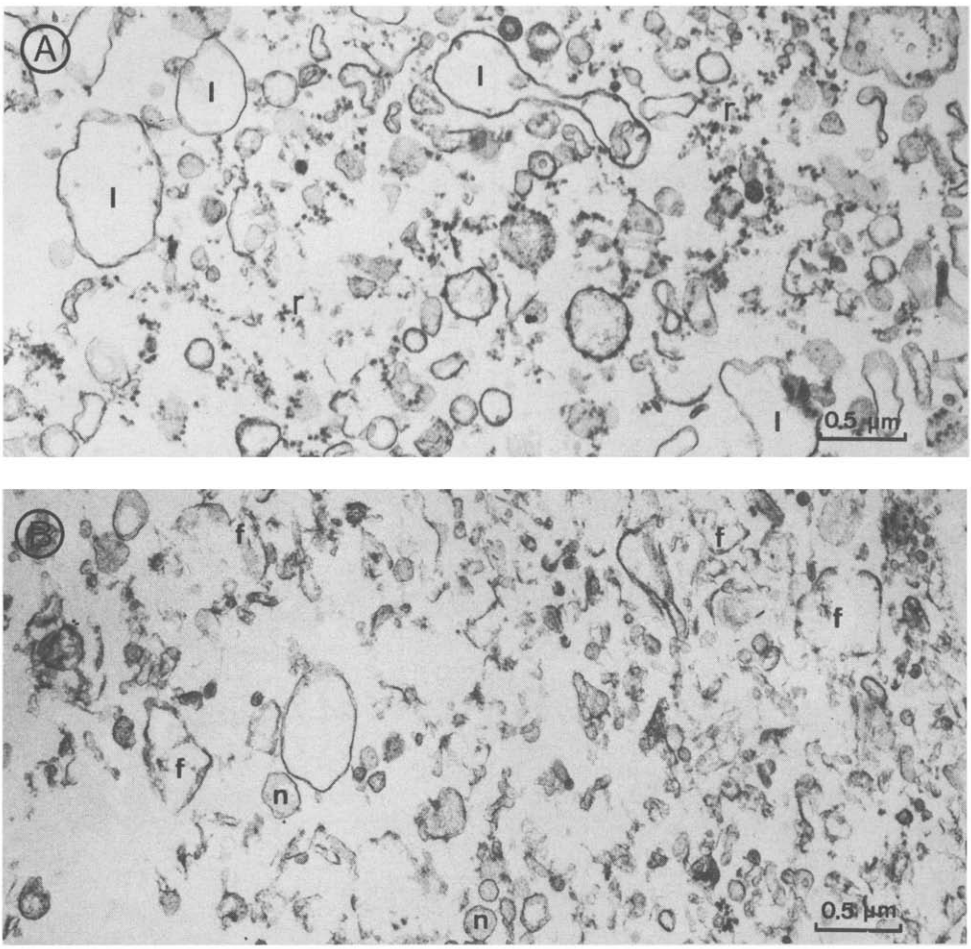

Fig. 2. Electron micrographs of thin sections of the P and MP fractions prepared from old fibroblasts. See Materials and Methods for the preparation and the embedding of the P and MP fractions. (A) Microsomal fraction containing rough and smooth vesicles. r, free ribosomes; l, large smooth vesicles. (B) MP fraction after digitonin treatment. f, some membranes with a special fenestrated appearance; n, others which are not fenestrated. Magnification, ×23 900.

0.5% for cytochrome oxidase and NADH:cytochrome *c* reductase, 1.8% for catalase, 2.9% for *N*-acetyl-β-D-glucosaminidase and 8% for galactosyltransferase.

As shown in Figs. 2 and 3, morphological analysis of these three fractions clearly indicates the evolution of the vesicles from a heterogeneous microsomal population containing rough and smooth membranes of different dimensions to the MP_1 fraction composed of relatively large fragments which present the typical fenestrated appearance of plasma membrane treated with digitonin [32]. The number of non-fenestrated membranes is significant in the MP fraction, but almost undetectable in the MP_1 fraction.

Comparison between plasma membranes from old and young cells

In order to investigate the effect of ageing on the plasma membrane, MP_1

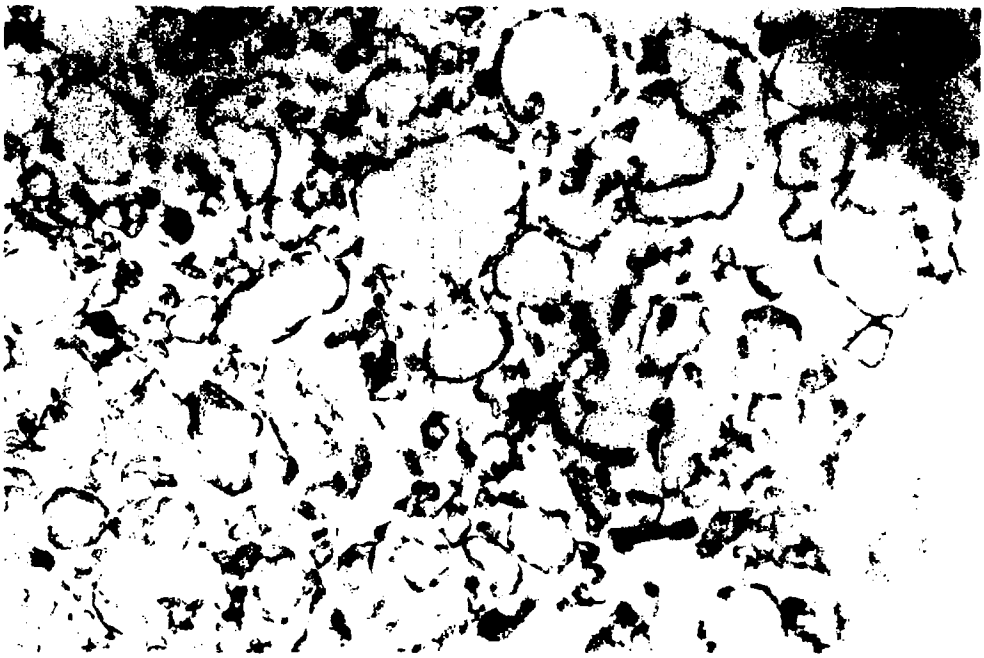

Fig. 3. Electron microscopy of thin sections of the MP_1 fraction prepared from old fibroblasts. See Materials and Methods for the preparation and the embedding of the MP_1 fractions. The MP_1 fraction contains mostly large fenestrated vesicles. n, small non-fenestrated vesicles. Magnification, X23 900.

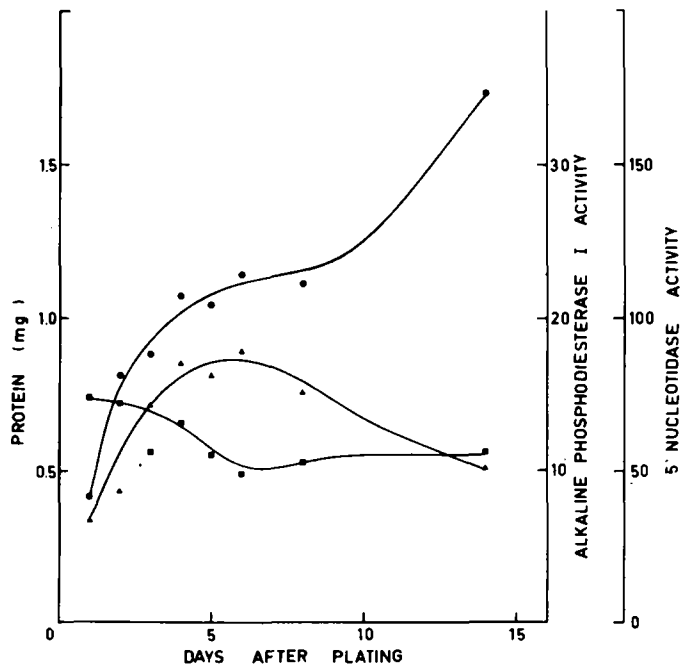

Fig. 4. Evolution of 5'-nucleotidase and alkaline-phosphodiesterase I activities following the plating of young cells. At day 0, cells from the population-doubling level of 23 were trypsinized and divided four times in 75-cm^2 flasks. Periodically, cells were harvested with a rubber policeman and assays were performed. Enzymatic activities are expressed as nmol of product liberated/min per mg of protein for (■) 5'-nucleotidase and (▲) alkaline phosphodiesterase I. (●) Amount of protein in each flask.

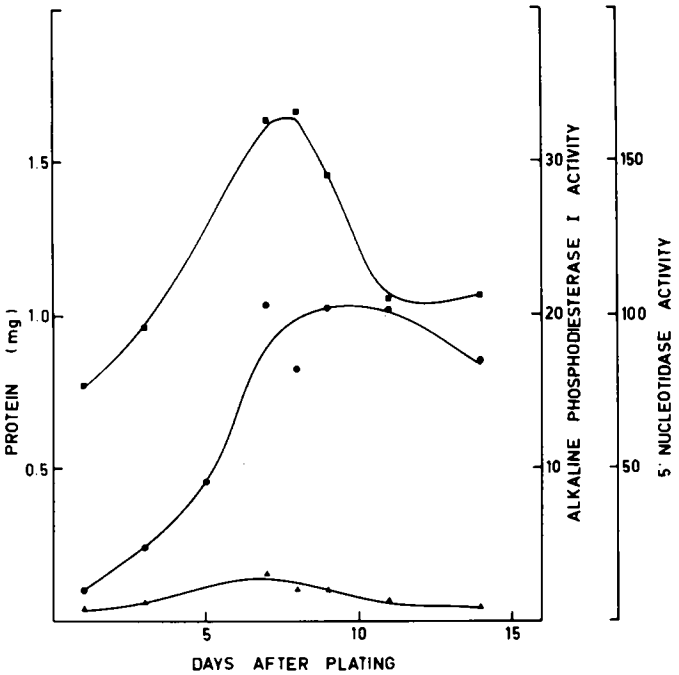

Fig. 5. Evolution of 5'-nucleotidase and alkaline-phosphodiesterase I activities following plating of old cells. The experiment was conducted as described in Fig. 4 but using cells with a population-doubling level of 50. (■) 5'-Nucleotidase and (▲) alkaline phosphodiesterase I. (●) Amount of protein in each flask.

fractions were prepared from young and old cell cultures following the method described above. No significant difference was observed in the evolution of the purification process of both preparations (not shown). The average relative specific activities of the two plasma membrane enzymes in the MP_1 fractions were 10.7 for young cells and 11.4 for old cells. This similar behaviour is understandable, since the physical parameters of the subcellular organelles in both cell types are very much alike [14]. The specific activities of 5'-nucleotidase and alkaline phosphodiesterase in the homogenates were, however, very much affected by the age of the cell cultures. The effects of the culture conditions were examined and are shown in Figs. 4 and 5. They distinctly influence the enzyme activity, but without masking the ageing effect.

Analysis of the MP and MP_1 fractions was performed by SDS-polyacrylamide gel electrophoresis. The distribution patterns are presented in Fig. 6. 31 constituents can be clearly observed in the MP preparations; five, labeled 1—5, disappear or are greatly reduced in the MP_1 preparations, probably resulting from the better purification process of the latter preparation. In both figures, the ageing process gives the same result: the number of membrane constituents is identical but in the preparations from old cells, one, labeled m, increases whereas four labeled, X, Y, W and Z decrease. The X protein has a molecular weight of 200 000 and its decrease, if not very large, was repeatedly found in three experiments. In order to estimate the influence of the culture conditions on these variations, plasma membranes from confluent and non-

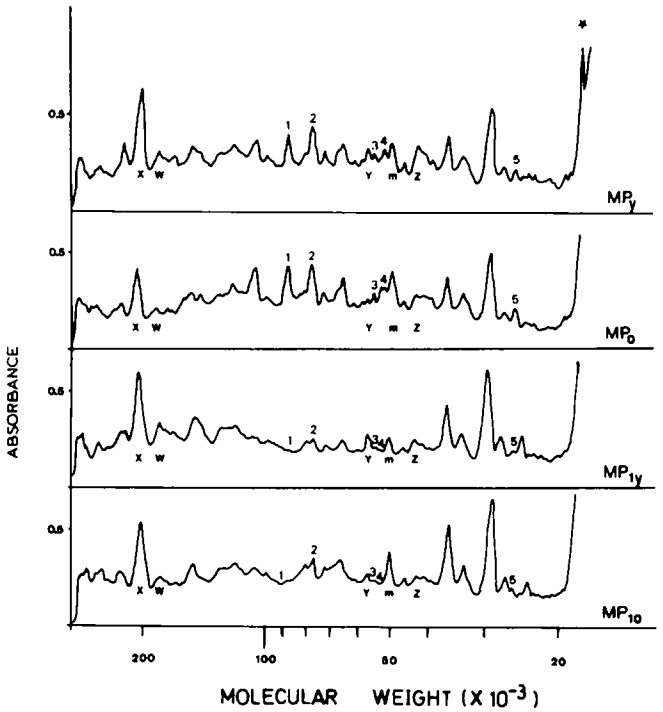

Fig. 6. Microdensitometer tracings of patterns obtained after electrophoresis on SDS-polyacrylamide gels of plasma membrane fractions MP and MP_1 from young (y) and old (o) cells. The fractions were prepared as described in Materials and Methods. Each sample contained 100 μg membrane protein. The gels were stained with Coomassie blue G 250. Star, peak of bromophenol blue. Components 1—5 absent or in very small amounts in the MP_1 fractions. X, W, Y and Z, components decreasing and m, increasing in the old cells.

confluent cells were prepared and analysed. As seen in Fig. 7, both preparations clearly show the differences already described between plasma membranes from young and old cells; the Y constituent seems to be particularly affected in non-confluent old cells.

Discussion

The purification of plasma membranes from fibroblasts is a difficult task, since they have to be separated from five other subcellular organelles having very similar properties. Based on the knowledge of the physical parameters of the subcellular organelles given in a previous analytical study [14] and taking advantage of the specific effect of digitonin on the plasma membranes (Fig. 1), we devised a three-step purification process which allows the preparation of a fraction containing 40% of the plasma membrane purified 12-fold compared to the homogenate (Table I). Each step is necessary to lower the contamination of one or two subcellular organelles: mitochondria and lysosomes in the first, peroxisomes in the second, endoplasmic reticulum and Golgi membranes in the third. Only the galactosyltransferase activity was still important in the MP_1 fraction; however, if it is definitely located in the Golgi appara-

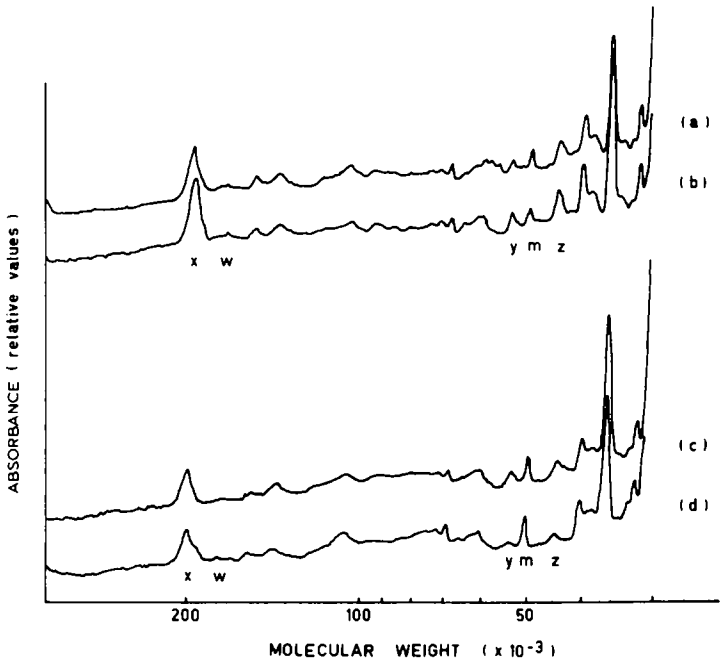

Fig. 7. SDS-polyacrylamide gel electrophoreses of MP_1 preparations prepared from young confluent (a) and non-confluent cell cultures (b), and from old confluent (c) and non-confluent (d) cell cultures. The letters, m, X, W, Y and Z, refer to peaks already described in Fig. 6. The fractions were prepared and analysed as described in Materials and Methods.

tus [33], Lamont et al. [34] suggest that it is also partly linked to the plasma membrane. From the biochemical analysis, especially from the very small amount of contaminant organelles, we can conclude that the MP_1 preparation is very pure. Morphological observations confirm the increasing purification of the plasma membrane in the three-step method, ranging from a heterogeneous population of vesicles in the P and MP fractions to a homogeneous population of fenestrated, mostly large vesicles in the MP_1 preparation.

The purity achieved by our preparation is also exemplified by the high relative specific activity of our enzyme markers compared to the values found in the literature which are usually much lower than 10 [35—38]. We observed an inactivation of 5'-nucleotidase and alkaline phosphodiesterase I in the homogenate due to the fact that cells were initially treated with a buffer containing 1 mM EDTA. It is well established that both these enzymes are inactivated in the presence of EDTA [39]. When this problem was not considered, artificially high relative specific activities were found for these enzymes (not shown) which could explain some of the published results [40].

The purification of 11—12-fold of the plasma membrane is small, however, when compared to values of 25—38 obtained on preparations of other cell types, like hepatocytes, and which contain the same amount of contaminant enzymes [31,41,44]. This discrepancy suggests that in fibroblasts, the plasma membrane represents a more important part of the total cell protein than in the other cell types. From our results, about 7—8% of the cell protein would

belong to the plasma membrane compared to only 3% in the hepatocyte. Unfortunately, no quantitative morphological study is at present available to support the calculations.

Using the purification method, we were able to compare plasma membranes from young and old cells. The behaviour of the membranes in the course of purification was identical for both cell types, but the relative content of their proteins varied. Reproducible differences in the relative amount of five membrane constituents were detected in the electrophoretic pattern (Fig. 6), but were not explained by the culture conditions (Fig. 7). Identification of the detected constituents with enzymes or identified proteins of the membrane was not carried out. Some of the proteins, especially in the 100 000—200 000 molecular weight range, could tentatively be compared to the findings of numerous glycoproteins having similar molecular weights [45,48]. The modification of 5′-nucleotidase and alkaline phosphodiesterase I activities were investigated. The differences between young and old cells are striking and cannot be accounted for by variations in the culture conditions (Figs. 4 and 5). Sun et al. [12] have followed the activity of 5′-nucleotidase during the cultivation of the fibroblasts and also found a large increase in the activity just before the end of the cell culture. These enzyme activities seem to be linked to the dynamism of the cells; their exact function is not known but Evans [49] suggested that they are involved in the degradation of the nucleotides which reach the cell membrane. In the case of 5′-nucleotidase, the increased activity correlates with the increase in nucleoside transport in aged cells [9], but this involvement would be difficult to conciliate with the decreased activity of alkaline phosphodiesterase I. It is interesting to note that after glucocorticoid stimulation of HTC cells, alkaline phosphodiesterase I and 5′-nucleotidase activities were modified in exactly opposite ways, like during the ageing process [50]. Three of the components, Y, m and Z, very much affected during ageing have a molecular weight similar to those modified after the glucocorticoid treatment of the cells [51].

Ageing affects the plasma membrane in a specific and quantitative way, since the relative amount of five components out of 26 is modified. However, our analyses represent an average behaviour of young or old cells. For example, in an old cell culture, the heterogeneity of the cell for their replicate rate is well established [52]. It is possible, therefore, that differences in ageing could be individually more pronounced than those observed here. The fact that the modifications are specific of only some components of the membrane suggests that they play a particular role in the behaviour of old cells. Some theories on the role of the plasma membrane in the ageing process have already been put forward based on the modification of one of their physiological functions [20]. From this work, we conclude that several modifications should be taken into account before a general view of the role of the plasma membrane in the aged cells can be developed.

Acknowledgements

The authors wish to thank Mrs. A. Deconninck and G. Detroye for their help in the preparation of the manuscript and Mrs. N. Fievet-Henry for her technical assistance; M.R. was an Aspirant of the F.N.R.S.

References

1 Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25, 585—621
2 Cristofalo, V.J. (1972) in Advances in Gerontological Research (Strehler, B.L., ed.), Vol. 3, pp. 45—79, Academic Press, New York
3 Hayflick, L. (1976) New Engl. J. Med. 295, 1302—1308
4 Strehler, B.L. (1977) Time, Cells, and Aging, p. 456. Academic Press, New York
5 Hayflick, L. (1977) in Handbook of the Biology of Aging (Finch, C.E. and Hayflick, L., eds.), pp. 159—186, Van Nostrand Reinhold Co., New York
6 Holliday, R. and Tarrant, G.M. (1972) Nature 238, 26—30
7 Milisauskas, V. and Rose, N.R. (1973) Exp. Cell Res. 81, 279—284
8 Bradley, M.O., Dice, J.F., Hayflick, L. and Schimke, R.T. (1975) Exp. Cell Res. 96, 103—112
9 Polgar, P., Taylor, L. and Brown, L. (1978) Mech. Aging Dev. 7, 151—160
10 Grinna, A.L.S. (1977) Gerontology 23, 342—349
11 Palumbo, M.E. (1979) Age 2, 1—4
12 Sun, A.S., Aggarwal, B.B. and Packer, L. (1975) Arch. Biochem. Biophys. 170, 1—11
13 Wang, K.-M., Rose, N.R., Bartholemew, E.A., Balzer, M., Berde, K. and Foldvary, M. (1970) Exp. Cell Res. 61, 357—364
14 Remacle, J., Houbion, A. and Houben, A. (1980) Biochim. Biophys. Acta 630, 57—70
15 Robbins, E., Levine, E. and Eagle, H. (1970) J. Exp. Med. 131, 1211—1222
16 Lipetz, J. and Cristofalo, V.J. (1972) J. Ultrastruct. Res. 39, 43—56
17 Bowman, P.D. and Daniel, C.W. (1975) Mech. Aging Dev. 4, 147—158
18 Kelly, R.O. and Skipper, B.E. (1977) J. Ultrastruct. Res. 59, 113—118
19 Van Gansen, P., Devos, L., Ozoran, Y. and Roxburgh, C. (1979) Biol. Cell. 34, 255—270
20 Zs-Nagy, I. (1979) Mech. Aging Dev. 9, 237—246
21 Hayflick, L. (1965) Exp. Cell Res. 37, 614—636
22 Cristofalo, V.J. and Sharf, B.B. (1973) Exp. Cell Res. 76, 419—427
23 Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. 61, 188—200
24 Baudhuin, P., Evrard, P. and Berthet, J. (1967) J. Cell Biol. 32, 181—191
25 Laemmli, U.K. (1970) Nature 227, 680—685
26 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321—349
27 Thinès-Sempoux, D., Amar-Costesec, A., Beaufay, H. and Berthet, J. (1969) J. Cell Biol. 43, 189—192
28 Emmelot, P., Bos, C.J., Benedetti, E.L. and Rumke, P.H. (1964) Biochim. Biophys. Acta 90, 126—145
29 De Duve, C., Wattiaux, R. and Baudhuin, P. (1962) Adv. Enzymol. 24, 291—358
30 Dillard, C.J. and Tappel, A.L. (1974) Biochem. Med. 11, 275—289
31 Fowler, S., Remacle, J., Trouet, A., Beaufay, H., Berthet, J., Wibo, M. and Hauser, P. (1976) J. Cell Biol. 71, 535—550
32 De Duve, C. (1971) J. Cell Biol. 50, 20—55$_D$
33 Morré, D.J., Merlin, L.-M. and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. 37, 813—819
34 Lamont, J.T., Thornton Gammon, M. and Isselbacher, K.J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1086—1090
35 Thom, D., Powell, A.J., Lloyd, C.W. and Rees, D.A. (1977) Biochem. J. 168, 187—194
36 Perdue, J.F. and Sneider, J. (1970) Biochim. Biophys. Acta 196, 125—140
37 Perdue, J.F., Kletzien, R. and Miller, K. (1971) Biochim. Biophys. Acta 249, 419—434
38 Branton, P.E. and Landry-Magnan, J. (1979) J. Cell. Physiol. 100, 159—168
39 Johnsen, S., Stokke, T. and Prydz, H. (1974) J. Cell Biol. 63, 357—363
40 Kartner, N., Alon, N., Swift, M., Buchwald, M. and Riordan, J.R. (1977) J. Membrane Biol. 36, 191—211
41 Coleman, R., Michell, R.H., Finean, J.B. and Hawthorne, J.N. (1967) Biochim. Biophys. Acta 135, 573—579
42 Touster, O., Aronson, N.N., Dulaney, J.T., Jr. and Hendrickson, H. (1970) J. Cell Biol. 47, 604—618
43 Jones, R.B., Staton, A.J. and Kiesow, L.A. (1973) Anal. Biochem. 55, 154—159
44 Brown, A.E., Lok, M.P. and Elovson, J. (1976) Biochim. Biophys. Acta 426, 418—432
45 Huang, C.C., Tsai, C.M. and Conellakis, E.S. (1973) Biochim. Biophys. Acta 332, 59—68
46 Gahmberg, C.G., Häyry, P. and Andersson, L.C. (1976) J. Cell Biol. 68, 642—653
47 Chen, K.Y., Kramer, R.H. and Conellakis, E.S. (1978) Biochim. Biophys. Acta 507, 107—112
48 Cohen, C.M., Kramer, R.H. and Branton, D. (1980) Biochim. Biophys. Acta 597, 29—40
49 Evans, W.H. (1974) Nature 250, 391—394
50 Rousseau, G., Amar-Costesec, A., Verhaegen, M. and Grammer, D.K. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1005—1009
51 Ivarie, R.D. and O'Farrell, P.H. (1978) Cell 13, 41—55
52 Macieira-Coelho, A. (1974) Nature 248, 421—422