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PLASMA MEMBRANE ISOLATED FROM ASTROCYTES IN PRIMARY CULTURES

ITS ACCEPTOR OXIDOREDUCTASE PROPERTIES

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Rat astrocytes in primary cultures were employed to isolate the plasma membrane. The method for the isolation of plasma membrane was based on the capacity of the cytoskeleton to adhere to the substratum entrapping intracellular organelles during freezing-thawing cycle performed on the cell. By washing the 'surface adherent framework', the untrapped plasma membrane were recovered and density equilibrium centrifugation resulted in the isolated membrane. The isolated plasma membrane was characterized on the basis of a variety of marker enzymes positive to the plasma membrane such as $(Na^+ + K^+)$-ATPase or 5′-nucleotidase as well as the lack of conventional markers of other endomembranes. Ultrastructurally the membranes, as isolated here, were mainly vesicular in nature. The isolated plasma membrane was devoid of the dehydrogenase responsible for NADH-cytochrome *c* reductase activity. However, NADH-ferricyanide reductase activity and the dehydrogenase system catalyzing the transfer of reducing equivalents from NADH or NADPH to dichloroindophenol seems plasma membrane redox system. The identical specific activity employing dichloroindophenol as an electron acceptor with NADH or NADPH as donor indicate a DT-diaphorase (EC 1.6.99.2) like activity in the astrocytes plasma membrane.

Introduction

Cell-cell and cell medium interactions involve plasma membrane. Hormonal receptor systems [1,2], ATPases [3,4], ion movement [5] and second messenger systems [6,7] are some of the means of communication taking place between the inside and outside of the cell. Plasma membrane is also involved in information storage and retrieval [8,9] in addition to a variety of functions, such as cell adhesion [10,11], contact inhibition [12,13], conduction of nerve impulses [14] and immunological defense [15]. The molecular events triggering such a communication is of particular importance in the understanding of oncogenesis [16,17] and the mode of action of protein kinase C [18]. In the channelization of cellular communication, either within the cell itself or with its environment, redox interactions are also possible. But how a cell senses redox system of another cell or that of its environment has not been clearly understood since the origin of plasma membrane dehydrogenase system [19] has remained less well characterized. The detailed knowledge of various redox systems located in plasma membranes from different cell types is desirable since it is becoming interestingly clear that the molecular assembly of one cell membrane varies from the other cell. Glial cell membranes

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are important because they facilitate the uptake of substances acting as synaptic transmitters [20,21]. Since the neurons and glia are intimately connected in the architectural network of brain and the heterogeneity of solid tissue as a starting material for the isolation of plasma membrane is not without pitfalls [22], the primary culture of astrocytes have been chosen to isolate glia cells plasma membrane. Broadly speaking there are two general approaches underlying the isolation of plasma membranes: the 'analytical' or 'preparative'. DePierre and Karnovsky [23] termed 'isolate and characterize' approach as the preparative one. In the present work the 'preparative' approach has been adopted and a battery of marker enzymes of cellular endomembrane systems were chosen to characterize the nature of the isolated plasma membrane from the cultured astrocytes.

The capacity of the cytoskeleton to adhere to the surface of the 'culture dish' entrapping intracellular organelles, after cycle of freezing and thawing cultured astrocytes have been exploited in the present study. The isolated plasma membrane was devoid of any microsomal or mitochondrial contamination. It does seem that astrocytes plasma membrane, as isolated here, is endowed with the dehydrogenase systems capable of catalyzing the transfer of reducing equivalents from NADH or NADPH to the artifical acceptors such as ferricyanide or dichloroindophenol. This is the first report where the isolation of cultured astrocyte plasma membrane is described and the isolated nerve cell membrane has been shown to have its own dehydrogenase system which acts exclusively on the external artificial acceptors.

Experimental Procedures

Radioactive [^{14}C]glucose 6-phosphate (57.4 mCi/mmol), [7-^{14}C]tyramine hydrochloride (56.0 mCi/mmol) and uridinediphosphate [^{14}C]galactose (302.0 mCi/mmol) were purchased from New England Nuclear. Na^{125}I (carrier free, 15 mCi/μg ^{125}I) was purchased from Radiochemical Center (Amersham, U.K.). Lactoperoxidase (EC 1.11.1.7), glucose oxidase (EC 1.1.3.4) adenosine 5'-triphosphate, adenosine 5'-monophosphate, glucose 6-phosphate, tyramine hydrochloride, uridinediphosphate galactose, acetylglucosamine, cytochrome *c* type VI, rotenone, NADH, NADPH (each 1 mg preweighed vials), 2,6-dichloroindophenol, were purchased from Sigma (U.S.A.); 2-mercaptoethanol was purchased from Fluka I.G., Buchs SC, (Switzerland); Triton X-100 and Dowex anion exchange resin from Serva Feinbochimia (F.R.G.); and, sodium deoxycholate, imidazole and glycylglycine from Merck (F.R.G.); ouabain (2% solution was purchased from Arnaud, France). Other chemicals and solvents were of Anal R grade and of highest purity.

Brain dissociation and cell cultures. Cerebral hemispheres of neonatal rats were excised, the meninges removed and the hemispheres thinly minced with scissors in 1% glucose tyrode solution. The tissue fragments were collected and dissociated mechanically by pipetting. The dissociated cells were transfered into Dulbecco's modified Eagle medium (Gibco) containing antibiotics (penicillin 10000 units, streptomycin 10000 units and fungizon 25 mg/ml and 20% fetal calf serum (Gibco). After centrifugation ($400 \times g$), the pellet was suspended in the same culture medium and seeded at a density of $0.75 \cdot 10^6$ cells per ml on 90 mm dishes (Falcon, U.S.A.). Cells were kept in 5% CO_2 atmosphere at 37°C and growth medium was changed at 5-day intervals with medium containing 10% fetal calf serum.

Enzymatic radioiodination. Lactoperoxidase catalyzes radioiodination of the proteins localized at the surface of the plasma membrane [24]. This was used to follow the plasma membrane fraction during the various steps of its isolation. Cells employed for enzymatic radioiodination were seeded on 35 mm diameter culture dishes (Falcon, U.S.A.) with a cell density similar to that of the other cell cultures. Two weeks old cell cultures were washed three times with Ca^{2+}-Mg^{2+} free phosphate-buffered saline (buffer I); 100 μCi Na^{125}I, 10 μg lactoperoxidase and 5 mU glucose oxidase were added. The iodination was initiated by the addition of 25 nmol D-glucose and the final volume of reaction mixture consisted of 0.4 ml of buffer I. The reaction was allowed to proceed for 10 min and the cells were washed seven times with buffer I (almost no radioactivity was obtained in the medium during the last washing).

Membrane preparation. Two weeks old astrocytes primary cultures were washed twice with

Tris-HCl buffer (50 mM, pH 7.6) and the washed cultured dishes were frozen at −20°C for 48 h. After thawing at room temperature, the surface adherent cellular material was washed twice with Tris-HCl buffer. These washing fractions (WF) were pooled together and centrifuged at 37 000 × *g* for 30 min. The supernatant was discarded and the pellet was suspended in 3 ml phosphate buffer (50 mM, pH 7.6). This suspension was layered on 33 ml of 33% sucrose (prepared in the phosphate buffer) and centrifuged at 105 000 × *g* for 3 h in a SW 27 rotor. The first 5 ml portion from the top of the centrifuge tube were discarded and the next two 3-ml fractions were diluted separately in 24 ml phosphate buffer and centrifuged at 37 000 × *g* for 1 h. The resulting pellets, from the two tubes (membrane fraction (MF)), were pooled together for morphological, chemical and biochemical characterization. The pellet obtained after 105 000 × *g* centrifugation was termed as pellet fraction (PF) and was suspended in appropriate medium for biochemical characterization.

Transmission electron microscopy. Cell cultures and pellet samples were fixed and embedded according to Porte et al. [25]. Thin sections, contrasted with uranyl acetate and lead citrate, were examined under a Siemens Elmiskop IA electron microscope.

Enzymatic assays. 5′-Nucleotidase (EC 3.1.3.5) was assayed according to Kai et al. [26] with the following modifications: (a) the final volume of the assay was 1 ml and contained 0.05% sodium deoxycholate per 50 μg protein; (b) the reaction was stopped by the addition of 1.7 ml isobutanol/benzene (1 : 1, v/v).

$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was assayed essentially as described by Post and Sen [27] with the modifications: (a) cellular material was homogenized in a Kontes minibomb cell disruption chamber at a protein concentration of 500 μg/ml for 5 min with a pressure of 60 bars, (b) the control assay was peorformed by incubating the disrupted cellular material in a buffer without NaCl, KCl or $MgCl_2$, (c) the final volume was maintained at 1 ml having NaF at a final concentratioan of 20 mM, (d) the reaction was stopped as in the case of the 5′-nucleotidase assay.

The assay of 5′-nucleotidase and $(Na^+ + K^+)$-ATPase activity was carried out on the membrane fraction (MF) or pellet fraction (PF). These fractions were suspended separately in Tris-HCl buffer and centrifuged at 37 000 × *g* for 30 min. It was repeated thrice. The pellet thus obtained did not contain any detectable inorganic phosphate.

Succinate dehydrogenase (EC 1.3.99.1) and monoaminooxidase (EC 1.4.3.4) were assayed according to King [28] and Fowler et al. [29], respectively.

Cytochrome *c* oxidase (EC 1.9.3.1) assay was performed as recommended by Smith [30]. Ferrocytochrome *c* was freshly prepared by dissolving oxidized cytochrome *c* in 0.1 mM phosphate buffer (pH 7.2) prebubbled with a stream of nitrogen and reducing it with a few crystals of dithionite. The excess dithionite was removed by pasing the sample through G-25 Sephadex column (25 × 3 cm). Cytochrome *c* was about 98% reduced and was immediately used.

NADH or NADPH acceptor:oxidoreductase activity was determined according to Crane and Low [31] at 30°C.

Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described by Swanson [32] with the following modifications: (a) cells were sonicated for 1 min. (Soni pre. 150 MSE, amplitude of 18 μm) and in the incubation medium (0.8 ml) 0.5 μCi of [^{14}C]glucose 6-phosphate at the final concentration of 10 mM was present, (b) the reaction was stopped with 1.4 ml isobutanol/benzene (1 : 1, v/v), mixed and centrifuged at 600 × *g* for 10 min, (c) after discarding the organic phase (upper phase), 400 μl of the aqueous phase was chromatographed on a Dowex column (2 ml bed volume) equilibrated with 100 μM sodium hydroxide. The phosphate free glucose was eluted in 1 ml distilled water; 12 ml Rotiszint scintillator (F.R.G.) were added, the sample was thoroughly mixed and the radioactivity was counted. The eluted material was free glucose, which was confirmed by thin-layer chromatography.

UDPgalactose: *N*-acetylglucosamine transferase (EC 2.4.1.38) was assayed according to Palmiter [33] as modified by Morré [34].

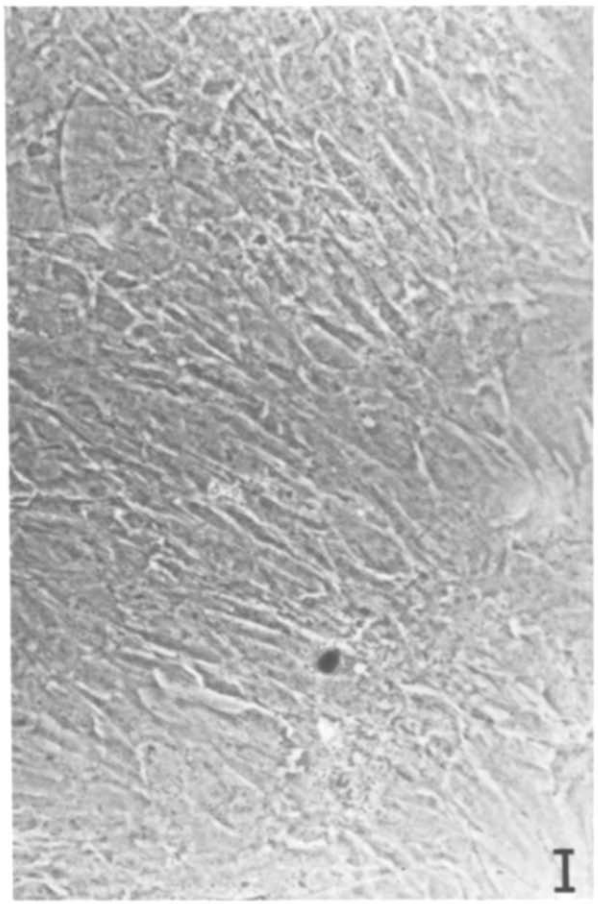
In order to avoid any discrepancy in the enzymatic determinations on account of freezing and thawing of the cells the enzymatic activity on frozen and thawed cells were considered as the activity in whole cells. Inorganic phosphate, which

was released by 5′-nucleotidase and $(Na^+ + K^+)$-ATPase, and protein was determined according to Martin and Doty [35] and Lowry et al. [36], respectively.

Results

When two weeks astrocytes primary cultures derived from neonatal rats (FIg. 1) were washed twice with Tris-HCl buffer (50 mM, pH 7.6) and frozen at −20°C for 48 h and thereafter thawed at room temperature, phase contrast microscopy revealed the presence of a 'surface' adherent framework', essentially composed of phase dark filaments (Fig. 2). Washing the 'surface adherent framework' either with Tris-HCl buffer or with phosphate buffer (50 mM, pH 7.6) did not alter its morphology. When similar treatment was carried out on 8-day-old cultures (from neonatal rats), only disrupted cell debris and not the surface adherent framework were seen (data not shown).

Prior freezing and thawing, cell cultures were labeled by the lactoperoxidase catalyzed radioiodination. The washed fraction of the 'surface adherent framework' was suspended in 3 ml phosphate buffer and was layered on the top of 33% sucrose and centrifuged at 105 000 × *g* for 3 h. The remaining 'surface adherent framework' was scrapped and treated likewise (the [^{125}I]iodine associated with this material was 12% of the total



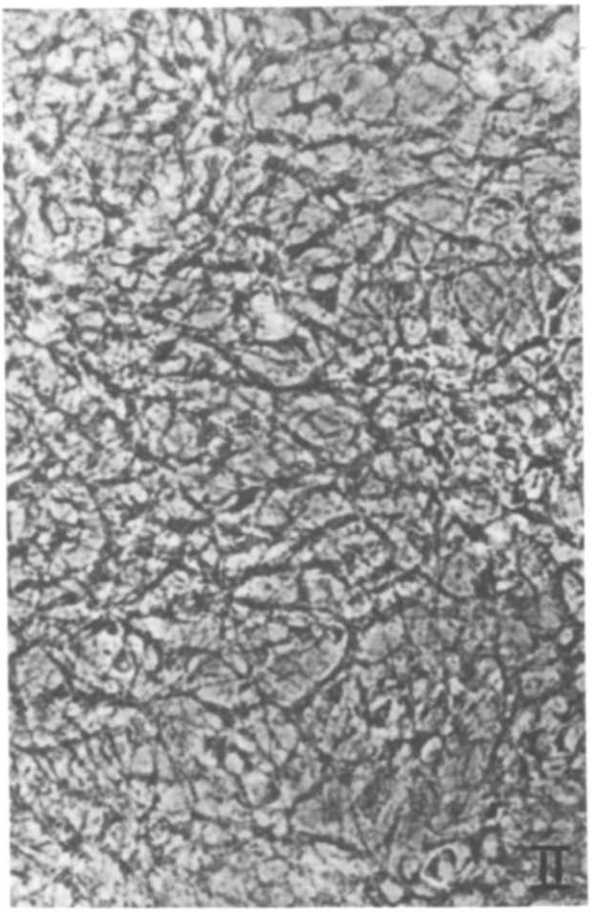


Fig. 1. Phase-contrast microscopy of 2-week-old astrocytes primary cultures. Monolayer flat polygonal cells are seen with fibrous mass (×125).

Fig. 2. Same as in Fig. 1, except the cells were frozen at −20°C for 48 h and subsequently thawed at room temperature. The observed phase dark filaments are essentially surface adherent framework (×250).

labeling). Analysis of the iodine labeled material of the washed fraction, obtained after density centrifugation (Fig. 3) showed that almost all the radioactivity was distributed between fractions numbering 5 to 11 counted from the top of the tube, whereas the radioactivity associated with the washed and scrapped 'surface adherent framework' was detected in the sediment. This clearly demonstrated that by freezing and thawing astrocytes primary cultures and by subsequent centrifugation, an enriched membrane material in the washing fraction (WF) was obtained.

Table I illustrates the distribution of the activity of plasma membrane and other endomembrane markers.

The distribution of $(Na^+ + K^+)$-ATPase as well as the 5'-nucleotidase in various cellular fractions, including that of the plasma membrane, followed similar pattern. However, the plasma membrane showed almost 20-fold rise in $(Na^+ + K^+)$-ATPase activity when compared to its activity in the whole cells, while 5'-nucleotidase was increased by 30-fold. None of the conventional markers activity belonging to mitochondria, microsomes and Golgi apparatus was observed in the isolated plasma membrane. Nonetheless, various endomembrane systems appeared to be associated with the 'surface adherent framework'.

When the enzymatic activity was observed in the membrane preparation from 8-day-old astrocyte cultures only a 3–6-fold enrichment in $(Na^+ + K^+)$-ATPase or 5'-nucleotidase activity was observed (data not shown).

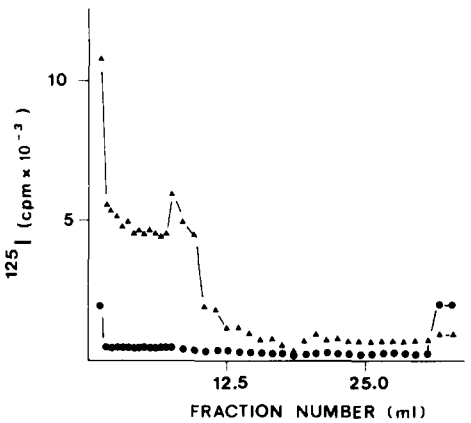


Fig. 3. Enzymatic (lactoperoxidase) radioiodination of two weeks astrocytes primary cultures, showing distribution of radioactivity parallel with the profile of density equilibrium centrifugation. Cells were frozen and thawed. Washed fraction (▲) and surface adherent framework (●) were layered on the top of 33% sucrose and centrifuged at 105000 × *g* for 3 h. Almost all the membranes enzymatically radioiodinated were found in the washed fractions numbering 5 to 11.

Table II illustrates the percentage of the recovered activity of various enzymes in different subcellular fractions. According to Lauter et al. [37] the total membrane protein only constituted one percent of the protein present in a single cell. The recovery of protein in isolated plasma membrane was only 0.10% which means that 10% of

TABLE I

SPECIFIC ACTIVITY OF VARIOUS MARKER ENZYMES IN DIFFERENT CELLULAR FRACTIONS

Frozen cells: Two weeks old astrocytes primary cultures were washed and frozen at −20°C for 48 h, thawed at room temperature and homogenized; SAF: surface adherent framework; PF: pellet fraction; MF: membrane fraction (plasma membrane isolated). These data are based on four sets of experiments. The standard deviation for highest value and for lowest value was ±7.0 and ±0.5, respectively.

	Specific activity (nmol·min^{-1}·(mg protein)$^{-1}$)			
	Frozen cells	SAF	PF	MF
$(Na^+ + K^+)$-ATPase	140.0	360	1000	3000
5'-Nucleotidase	3.0	6.0	30.0	65.0
Cytochrome *c* oxidase	7.1	19.8	17.8	NIL
Succinate dehydrogenase	17.0	17.0	0.05	NIL
Monoaminooxidase (MAO)	13.0	16.0	5.0	NIL
Glucose-6-phosphatase	56.0	42.0	3.0	NIL
NADPH-cytochrome *c* reductase	20.0	8.0	3.5	NIL
UDP galactose: *N*-acetylglucosamine transferase	2.0	2.0	n.d.	NIL

TABLE II

PERCENTAGE DISTRIBUTION OF ENZYMIC MARKERS IN VARIOUS CELLULAR FRACTIONS

The total recovery represents the total amount of the enzyme activity recovered in three fractions relative to the amount of particular enzyme in the whole homogenate; SAF: surface adherent framework; PF: pellet fraction; MF: membrane fraction (plasma membrane isolated).

	SAF	PF	MF	Total recovery
Protein	90.0	10.0	0.1	100.0
$(Na^+ + K^+)$ATPase	64.0	15.0	15.0	94.0
5′-Nucleotidase	70.0	18.0	12.0	90.0
Cytochrome *c* oxidase	67.0	25.0	0.0	92.0
Succinate dehydrogenase	95.0	5.0	0.0	100.0
Monoaminooxidase	93.0	0.7	0.0	93.7
Glucose-6-phosphatase	75.0	3.0	0.0	78.0
NADPH-cytochrome *c* reductase	90.0	3.5	0.0	93.5
UDP galactose: *N*-acetylglucosamine transferase	100	0.0	0.0	100.0

the membrane proteins were recovered in the isolated membrane. This was further supported by the $(Na^+ + K^+)$-ATPase and 5′-nucleotidase recovery in the plasma membrane fraction, i.e., 15% and 12%, respectively. The major portion of the activity of these plasma membrane markers was found in the 'surface adherent framework' indicating that only a small part of the membrane could be separated and purified; a large proportion of the membrane still remained adhered to the substratum. This is also significant that all the marker enzymes negative in terms of plasma membrane were recovered mainly with the 'surface adherent framework'.

Table III outlines the presence of NADH (NADPH):acceptor oxidoreductase activity in cell homogenate as contrasted from the isolated plasma membrane. The cell homogenate contained both rotenone insensitive (microsomal) and rotenone sensitive (mitochondrial) NADH cytochrome *c* reductase activity. However, the isolated plasma membrane did not show any dehydrogenase activity employing natural acceptor like cytochrome *c* or metabolic acceptor such as glyoxylate. It is most interesting that the isolated plasma membrane only showed dehydrogenase activity catalyzing the transfer of electrons from NADH or NADPH to artifical acceptors. The NADH-ferricyanide reductase as well as NADH dichloroindophenol reductase was present while dehydrogenase activity responsible for NADPH ferricyanide reductase was totally absent.

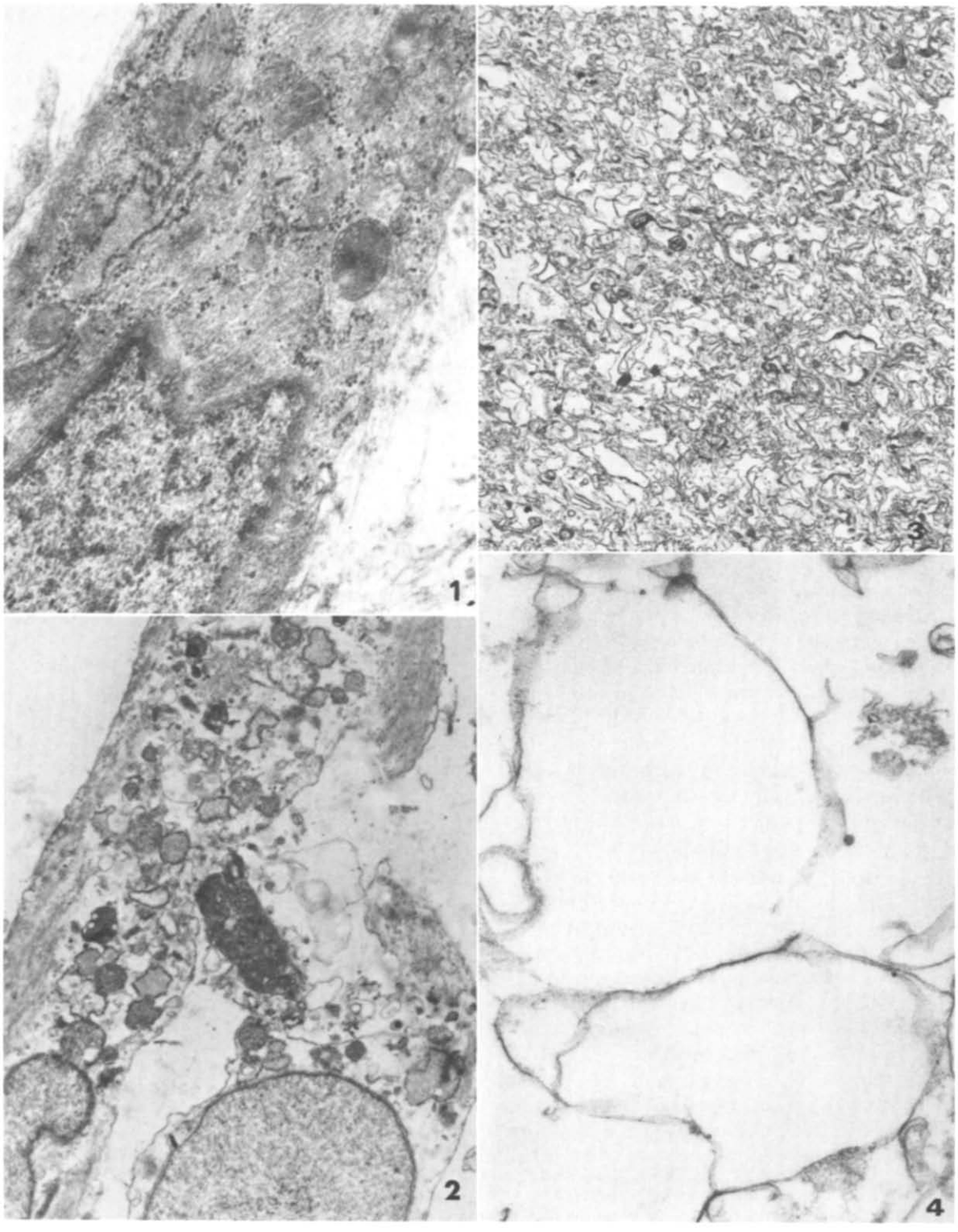
In order to study the effect of the freezing

TABLE III

NADH (NADPH) ACCEPTOR OXIDOREDUCTASE ACTIVITY IN ISOLATED PLASMA MEMBRANE AND IN CELL HOMOGENATES OF ASTROCYTES

50 mM phosphate buffer (pH 7.0). The final volume of the reaction mixture was 1.0 ml. NADH (100 μM), NADPH (100 μM), glyoxylate (4 mM), ferricyanide (100 μM), 2,6, dichloroindophenol (100 μM). In cytochrome *c* reductase assay 3 mM sodium cyanide was present. Rotenone (4 μM) was dissolved in 95% ethanol. The cell homogenate was made by homogenizing the frozen and thawed cells in a Kontes minibomb cell disruption chamber. The protein content of the cell homogenate in each assay was 50–60 μg/ml and in the case of plasma membrane the protein ranged between 10–20 μg/ml. Cell homogenate: the cells were frozen as in Table I and harvested with appropriate buffer and homogenized in a Kontes minibomb cell disruption chamber for 5 min at 60 bars pressure. The data are based on four different experiments. The standard deviation for high value and for low value was ±4 and ±0.5, respectively.

Acceptor oxidoreductase	Specific activity ($nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$)	
	Cell homogenate	Plasma membrane
NADH cytochrome *c* reductase		
minus rotenone	12.0	NIL
plus rotenone	6.0	NIL
NADPH cytochrome *c* reductase	20.0	NIL
NADH glyoxylate reductase	38.0	NIL
NADH ferricyanide reductase	49.0	50.0
NADPH ferricyanide reductase	9.0	NIL
NADH dichloroindophenol reductase	15.0	60.0
NADPH dichloroindophenol reductase	17.0	60.0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process on the cell cultures and to characterize the membrane fraction, ultrastructural studies were performed on living cells, frozen cells and the membrane fractions (Fig. 4). Astrocytes in our culture conditions conserved their usual ultrastructural characteristics. The cytoplasm contained a few marginal elongated rough endoplasmic reticulum cisternae which was filled with amorphous material (Fig. 4.1). Numerous free ribosomes were seen scattered among the abundant filaments occupying the major part of the cytoplasm. Relatively few mitochondria and dense lysosomal bodies were seen in certain cells (Fig. 4.1).

After freezing, the most obvious change was the clearing up of the cytoplasmic matrix and the condensation of the packed filaments in marginal cytoplasmic areas (Fig. 4.2). The rough endoplasmic reticulum was fragmented assuming the shape of round cavities like structure also containing electron dense material. The wall of these cavities were covered with ribosomes. Mitochondria, generally swollen, were recognizable by the presence of cristae. The plasma membrane was often partially disrupted, but in the majority of cases, well preserved membrane was seen around the cell.

The pellet fraction (PF) revealed the presence of damaged membrane structures along with intracellular components including some damaged mitochondria (figure not shown).

The membrane fraction, i.e., the isolated plasma membrane-demonstrated a homogenous preparation (Fig. 4.3). Occasionally a few cytoplasmic residues were seen. No cytoplasmic organelles such as mitochondria or endoplasmic reticulum could be detected. In addition to membranes, dense packed filaments (glial filamentous material) were always found. Membranes were largely in a vesicular form (Fig. 4.4). Delimitable elongated saccules tightly sticking to each other were also seen.

Discussion

Primary cell cultures derived from mechanically dissociated brain hemispheres of neonatal rats have been shown in our culture conditions to contain essentially astrocytes [38]. The plasma membrane of astrocytes (in culture) was isolated, in the present study, avoiding homogenization or stabilization of cell membrane. It is common knowledge that membrane stabilization prior actual isolation step adversely affects the activity of enzymes associated with membranes [39]. The method employed here was based on the fact that during the freezing-thawing cycle the cytoskeleton is able to entrape intracellular organelles. The 'surface adherent framework' obtained as a sequel of freezing-thawing operation was in fact cell cytoskeleton [40] associated with other organelles. But a part of the plasma membrane remained untraped during freezing-thawing and it was possible to recover the untraped plasma membrane by simply washing the 'surface adherent framework'. The underlying principle of the attachment of the cytoskeleton to the substratum was supported by the observation that freezing and thawing 8-day-old astrocytes did not produce 'surface adherent framework'. The isolation of plasma membrane was not achieved when 8-day-old cultures were used, since development of cytoskeleton required two weeks in culture [38,41].

The recovery of 88% radioactivity in the fraction containing plasma membrane did not mean that the 88% of the plasma membrane of the cell was recovered. This amount only represented that portion of the plasma membrane which was accessible to the enzymatic radioiodination. On the basis of recovery of protein or recovery of plasma membrane marker enzymes it may be argued that only 10–15% of the plasma membrane actually underwent radioiodination. Only 0.1% of the pro-

Fig. 4. (1) Electron micrograph showing typical aspect of astrocyte in cultures. Note the marginal rough endoplasmic reticulum cisternae filled with amorphous material. Numerous free ribosomes; abundant diffused filaments scattered over the cytoplasmic space are seen (×40000). (2) Electron micrograph of astrocytes immediately after freezing and thawing. Rough endoplasmic reticulum cisternae are fragmented into round cavities embedded with ribosomes. Mitochondria are yet visualized. Filaments are condensed marginally on the cytoplasmic matrix. (×20000). (3) Electron micrograph of isolated plasma membrane. Note the homogeneity of the membrane preparation constituting almost exclusively membranous structures. No intracellular organelles are seen (×7700). (4) Ultrastructural detail of the plasma membrane showing usual membrane aspect, i.e., tightly accolated and delimiting saccular clear spaces (×41500).

tein was recovered in the isolated membrane which actually represented 10% of the theoretical membrane protein content of the cell.

In terms of fold of enrichment of the plasma membrane markers in isolated membrane, 20-fold enrichment in $(Na^+ + K^+)$-ATPase and 30-fold rise in 5′-nucleotidase specific activity was observed. The variation in the two plasma membrane conventional markers may be attributed to the difference in the assay conditions of the two enzymes. The assay of 5′-nucleotidase was carried out in the presence of deoxycholate.

The ultrastructural studies revealed homogenous distribution of plasma membrane structures in the isolated membrane fraction (Figs. 4.3 and 4.4). Although intracellular components were devoid in this fraction, still certain not well defined cellular material were occasionally seen during electron microscopic examination. However, the association of cytoskeletal network with the various plasma membranes [43] particularly the plasma membrane derived from the cells of central nervous system is not uncommon [44]. This is commonly experienced that the cytoskeletal actin is in intimate contact with the inner side of the plasma membrane [43] and howsoever vigorous method may be invoked to disrupt this contact, still a residual part remains intact. Nevertheless, the presence of a scattered cytoskeletal element occasionally seen during electron microscopic examination in the isolated plasma membrane did not mar the purity of the isolated membrane.

The electron microscopic examination of surface adherent framework clearly showed that most of the intracellular organelles were attached to the substratum (Fig. 4.2). This has been further supported since all the mitochondrial markers such as the activity of cytochrome *c* oxidase or succinic dehydrogenase were identified with the 'surface adherent framework'. Similarly, NADPH-cytochrome *c* reductase or glucose-6-phosphatase activity were also present in this fraction, demonstrating the association of microsomes with this fragment. However, the pellet fraction, i.e., the pellet obtained after density centrifugation at $105\,000 \times g$ appeared to contain some inner mitochondrial membrane since cytochrome *c* oxidase activity was associated here. We are conscious of the encountered difficulty in assaying glucose-6-phosphatase in the brain cell homogenate [45]. This was circumvented here using radioactive glucose 6-phosphate and isolating the product employing Dowex anionic column exchanger.

To the best of our knowledge a systematic study of various dehydrogenases in the cell homogenates of cultured astrocytes have not been reported earlier. Here a number of NADH (or NADPH) acceptor:oxidoreductases activity were analysed in the cellular homogenate. Both rotenone sensitive as well as rotenone insensitive NADH-cytochrome *c* reductase activity were present. This clearly showed that the dehydrogenase system having mitochondrial as well as non mitochondrial origin were located in these cells. Similarly, the dehydrogenase system capable of metabolizing glyoxylate, a metabolic electron acceptor, was also present. However, NADPH was unable to reduce glyoxylate showing that the dehydrogenase system responsible for transfer of reducing equivalents from NADPH to glyoxylate was absent. When ferricyanide or dichloroindophenol was employed as electron acceptor both NADH- or NADPH-linked dehydrogenases seem active.

The origin of dehydrogenase system of the plasma membrane has remained obscure. Nevertheless, overwhelming evidence have appeared strongly favouring that the plasma membrane has its own dehydrogenase system distinct from the dehydrogenases of the inner mitochondrial membrane or the membrane of endoplasmic reticulum origin [46]. It has been observed here that NADH-cytochrome *c* reductase was not present in astrocytes plasma membrane. In contradistinction, it was observed earlier [18] that plasma membranes isolated from the outer membrane of synaptosomes showed higher NADH-cytochrome *c* reductase activity. It is interesting to note that astrocytes membrane and synaptosomal membrane derived from the nervous system were endowed with different dehydrogenase so far as NADH-cytochrome *c* reductase was concerned. Recently, Malviya et al. [47] demonstrated the presence of this enzyme system in the plasma membrane isolated from the chromaffin cells of bovine adrenal medulla. It may be argued that the location of this NADH dehydrogenase differs from one plasma membrane to the other. NADH dehydrogenase activity employing ferricyanide or fer-

ricytochrome *c* as the electron acceptor were studied in the erythrocyte plasma membrane from a variety of species [48]. These workers observed that the activity with the two acceptors were not parallel: the NADH-ferricyanide reductase activity was always higher than the NADH-cytochrome *c* reductase. Moreover, the complete absence of NADH dehydrogenase responsible for NADH-cytochrome *c* reductase activity in the isolated astrocytes plasma membrane and the presence of the dehydrogenase system capable of electron transfer from NADH to ferricyanide or dichloroindolphenol suggest that this may be due to differences in the actual electron carrier components. Cytochrome *b* type may be a strong candidate to account for this difference. Whether astrocytes plasma membrane is endowed with this redox protein remains to be ascertained. It has been shown that the neutrophil plasma membrane [49], chromaffin cell plasma membrane [47] or red cell membrane [50] contain *b* type cytochrome.

When the NADH or NADPH dehydrogenases were assayed on the isolated astrocytes membrane employing artificial acceptors such as ferricyanide or 2,6-dichloroindophenol still more interesting observations were highlighted. With ferricyanide as an acceptor NADPH dehydrogenase activity was absent while NADH dehydrogenase activity was present. But the dehydrogenase system catalysing both NADH or NADPH-dichloroindophenol reductase was present in the isolated plasma membrane of astrocytes. These data do favour the assumption that the activity associated with these dehydrogenases was due to their occurence in the isolated plasma membrane and not as a consequence of contamination due to other endomembranes. Moreover, identical specific activity of the dehydrogenase catalyzing NADH-dichloroindophenol reductase and NADPH-dichloroindophenol reductase indicated DT-diaphorase (EC1.6.99.2)-like activity. The possible role of the plasma membrane dehydrogenase during onset of density-dependent inhibition of growth is actually under investigation.

An organism communicates with its external environment through the nervous system and molecular assembly of the plasma membrane enveloping the nervous cell may play an important role in the phenomenon of information transfer. The presence of NADH-ferricyanide reductase or NADH (NADPH)-dichloroindophenol reductase in the plasma membrane of nervous cell might provide additional clues to the molecular understanding of information transfer between the external environment and internal medium at the cellular level.

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