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ISOLATION OF PLASMA MEMBRANES FROM MURINE EPENDYMOBLASTOMA AND SUBCELLULAR DISTRIBUTION OF AMPHOTERICIN B IN THIS TUMOR

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Summary

A method for the isolation of plasma membranes from an experimental murine ependymoblastoma is described. In this procedure, 5'-nucleotidase was used as the plasma membrane marker, since cytochemical methods demonstrated that the enzyme was present on this subcellular structure only.

The final plasma membrane preparation showed a 15-fold enrichment in 5'-nucleotidase activity and a 17-fold enrichment in the activity of phosphodiesterase I, another plasma membrane marker. The specific activity of β-glucuronidase (lysosomal enzyme) was twice that of the whole homogenate, the specific activity of arylesterase (microsomal enzyme) was similar to that of the whole homogenate and succinate dehydrogenase (mitochondrial marker) was not detected. Electron microscopy of this fraction showed vesicles on which 5'-nucleotidase activity could be demonstrated.

The subcellular distribution of [^{3}H]amphotericin B per mg of protein was similar in the plasma membrane preparation and in the whole homogenate. It is concluded that, in ependymoblastoma, amphotericin B shows no selective affinity for the plasma membrane.

Introduction

The transplantable murine ependymoblastoma, which derived from the tumour originally induced with methylcholanthrenen by Zimmerman [1], shares with human gliomas morphological similarities, as well as some metabolic ones [2] and sensitivity to chemotherapeutic agents (mainly the nitrosourea derivatives) [3]. This model appears therefore to be suitable not only for the screen-

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ing of new drugs active against cerebral neoplasms, but also for investigations of their mode of action.

The purpose of this report is to describe a method for the isolation of plasma membranes from mouse ependymoblastoma and to describe some biochemical characteristics of this subcellular fraction. This work was also undertaken in order to study the subcellular distribution of amphotericin B, which potentiates the therapeutic effect of a nutrosourea on the mouse ependymoblastoma [4]. Indeed, this potentiation could result from an enhanced permeability due to the binding of Amphotericin B to the cholesterol of plasma membrane.

Materials and Methods

Animals and tumors

The ependymoblastoma 01B111 used in this study was maintained in vivo by subcutaneous transplantation. Mice carrying the ependymoblastoma were purchased from Hazleton Laboratories (Vienna, Va). In our laboratory, the tumor was transplanted subcutaneously into the flank of male C57 Bl mice (Charles River Laboratories, North Wilmington, Mass.), using a previously described procedure [5]. The ependymoblastomas were taken for subcellular fractionation 12—15 days after transplantation, when they weighed about 1 g.

Subcellular fractionation

The animals were killed by cervical dislocation and the tumors were quickly removed and rinsed in ice cold bicarbonate buffer ($NaHCO_3$ 1 mM, $CaCl_2$ 0.5 mM, pH 7.5). All subsequent manipulations were performed at 4° C. About 8 g of tumor tissue were weighed and homogenized by 20 strokes in a Dounce homogenizer, fitted with pestle A (Kontes Glass Co., Vineland, N.J.), in 10 vols. of the bicarbonate buffer. The homogenate was filtered through four layers of cheesecloth (16 mesh ASTM), diluted to 100 times the weight of tissue and spun for 5 min at 800 × *g* (the values given for centrifugal force correspond to the average radius).

The SI supernatant, obtained after the first centrifugation, was centrifuged for 30 min at 16 000 × *g* in a Spinco model L2-65B, using the angular rotor 21. The pellets P2, collected after this centrifugation, were suspended in 16 ml of bicarbonate buffer and the suspension was mixed with an equal weight of 46% sucrose (w/w).

The mixture was laid on four continuous sucrose gradients of density 1.12 to 1.25. After a 120 min centrifugation at 64 000 × *g* using a SW27 rotor, a distinct fraction was seen in the gradient as a band corresponding to a sucrose density of about 1.13; this fraction was enriched 9-fold in 5′-nucleotidase (Fig. 1). This material was removed with a syringe, mixed with 65% sucrose (w/w) to obtain a sucrose concentration of 45% and placed on the bottom of two SW27 tubes. Over each fraction 8 ml of 41% sucrose (w/w) (d = 1.19), 7 ml of 33% sucrose (w/w) (d = 1.15), and finally 7 ml of 23% sucrose (w/w) (d = 1.10) were layered. The tubes were spun for 12—15 h at 64 000 × *g*. The 5′-nucleotidase-enriched layer appeared at the interface d-1.10/d 1.15. It was removed with a syringe, diluted with bicarbonate buffer and centrifuged for 50 min at 76 000 × *g*. The pellet, considered as the final plasma membrane preparation, was suspended in the bicarbonate buffer.

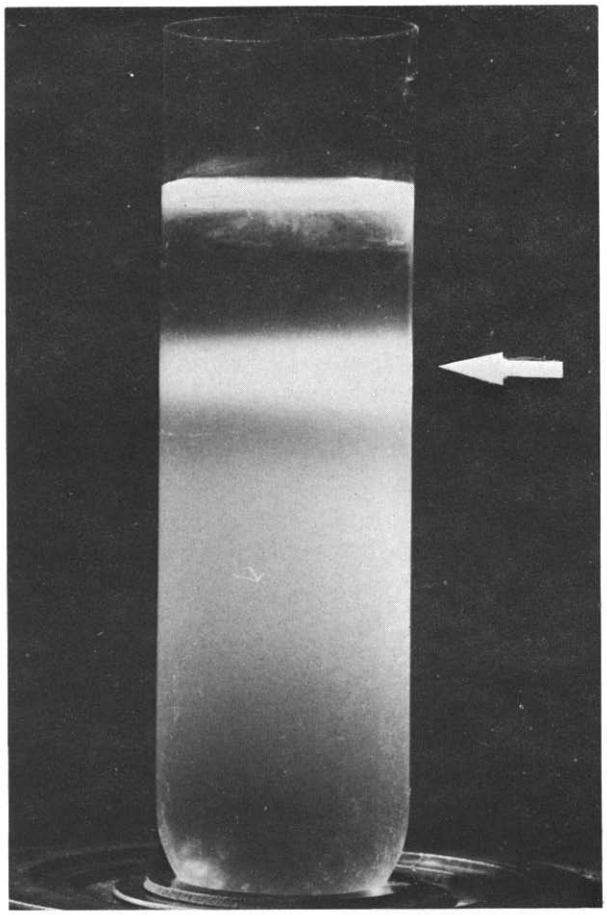

Fig. 1. Isolation of plasma membranes by isopycnic sucrose sedimentation. The P_2 pellet was centrifuged for 120 min at 64 000 X g in a sucrose gradient of density 1.12—1.25. The membrane-enriched fraction appears as a distinct band in the gradient (arrow).

Morphological studies

For electron microscopy, fragments of the plasma membrane pellet were fixed in 2.5% cacodylate-buffered glutaraldehyde for 90 min at 4°C. After a single washing with cold buffer, the samples were post-fixed in 2% cacodylate-buffered OsO_4 for 2 h at 4°C, dehydrated in graded cold ethanols and embedded in Epon.

For the cytochemical demonstration of 5′-nucleotidase, small blocks of ependymoblastoma were fixed at 4°C in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 90 min. After 6 washings in cold buffer over a period of 60 min, the samples were incubated at 37°C for 60 min in a medium containing: 60 mM Tris-maleate, 1.5 mM $Pb(NO_3)_2$, 5 mM $Mg(NO_3)_2$, 1.3 mM 5′-AMP buffered at pH 7.5. After a rapid washing in cold buffer, the reaction product was converted into lead sulfide by immersion in ammonium sulfide. The samples were then washed again in cold buffer, post-fixed in 2% cacody-

late-buffered OsO_4 for 2 h at 4°C, rapidly dehydrated in cold ethanols and embedded in Epon.

Ultra-thin sections were studied on a Siemens Elmiskop I electron microscope with and without uranyl acetate and lead citrate staining.

The following control experiments were performed: incubation in substrate-free medium, in medium without $Pb(NO_3)_2$ or without $Mg(NO_3)_2$.

The fixation and incubation procedures used for the demonstration of 5'-nucleotidase in the plasma membrane enriched fraction were performed following the same procedures.

Enzymatic assays

5'-Nucleotidase (EC 3.1.3.5), succinate dehydrogenase (EC 1.3.99.1), β-glucuronidase (EC 3.2.1.31) and phosphodiesterase I (EC 3.1.4.1) were assayed following previously published procedures.

Each incubation tube contained 1.5 mg protein of the whole homogenate for the estimation of 5'-nucleotidase [6], 0.4 mg protein for succinate dehydrogenase [7], for β-glucuronidase [8] and phosphodiesterase I [9]. In the assay of the latter enzyme, the incubation time of 10 min used in the original procedure was lengthened to 60 min.

For the assay of arylesterase (EC 3.1.1.2) the incubation medium contained 2.7 μmol EDTA, 2.7 μl Triton X-100, 54 μmol phosphate buffered to pH 7.4, and 0.4 mg protein in a total volume of 2.95 ml. The reaction was initiated with 0.05 ml of *o*-nitrophenyl acetate, 180 mM, in methanol. The activity of esterase was estimated by measuring for 10 min at room temperature the increase of absorbance at 420 nm. The rate of *o*-nitrophenol liberation was calculated by assuming an extinction coefficient of 2417 $M^{-1} \cdot cm^{-1}$ for this compound.

Chemical determinations

Proteins were estimated by the method of Lowry [10], using bovine serum albumin as a standard. Nucleic acids were extracted by the Schneider procedure and DNA estimated by the method of Dische [11]. Total lipids were extracted and washed following the procedure of Folch et al. [12]. Free and total cholesterol were determined by the method of Sperry and Webb [13]. The amount of phospholipids was calculated by multiplying by 25 the value of phosphorus measured by the method of Fiske and SubbaRow [14].

Subcellular distribution of [^{3}H]amphotericin B

Just before use, the drug was dissolved in 5% glucose containing 2 mg/ml deoxycholate. Each mouse received 0.1 mCi of the labeled compound intraperitoneally.

For the measurement of radioactivity, 0.25 ml of each subcellular fraction was treated at 50°C. by 1.5 ml of NCS (Nuclear Chicago Solubilizer, Amersham Searle, Arlington Heights, Ill.). After cooling at room temperature, 10 ml of a toluene scintillation fluid were added and the radioactivity measured by scintillation counting. Efficiency was estimated by the internal standard method.

Reagents and chemicals

NH_4-*p*-nitrophenyl-5′-thymidilate was purchased from Merck (Darmstadt, G.F.R.) and *p*-nitrophenyl-β-D-glucuronide from Boehringer (Mannheim, G.F.R.). All other substrates came from Sigma (St. Louis, Mo.). [^{3}H]Amphotericin B (spec. act. 0.2 Ci/g) was obtained through the courtesy of Dr. Lecocq (Squibb Laboratories, Belgium) and prepared by Mr. Wynand (I.R.E., Fleurus, Belgium).

Results

Cytochemical localization of 5′-nucleotidase

Cytochemical reaction of 5′-nucleotidase was restricted to the cell periphery (Fig. 2), demonstrating that in mouse ependymoblastoma this enzyme is localized to the plasma membrane. Negative cytochemical reactions were observed in control incubations where the addition of 5′-AMP or Pb^{2+} was omitted. However, unlike in rat lymphocytes [15], the absence of exogenous Mg^{2+} did

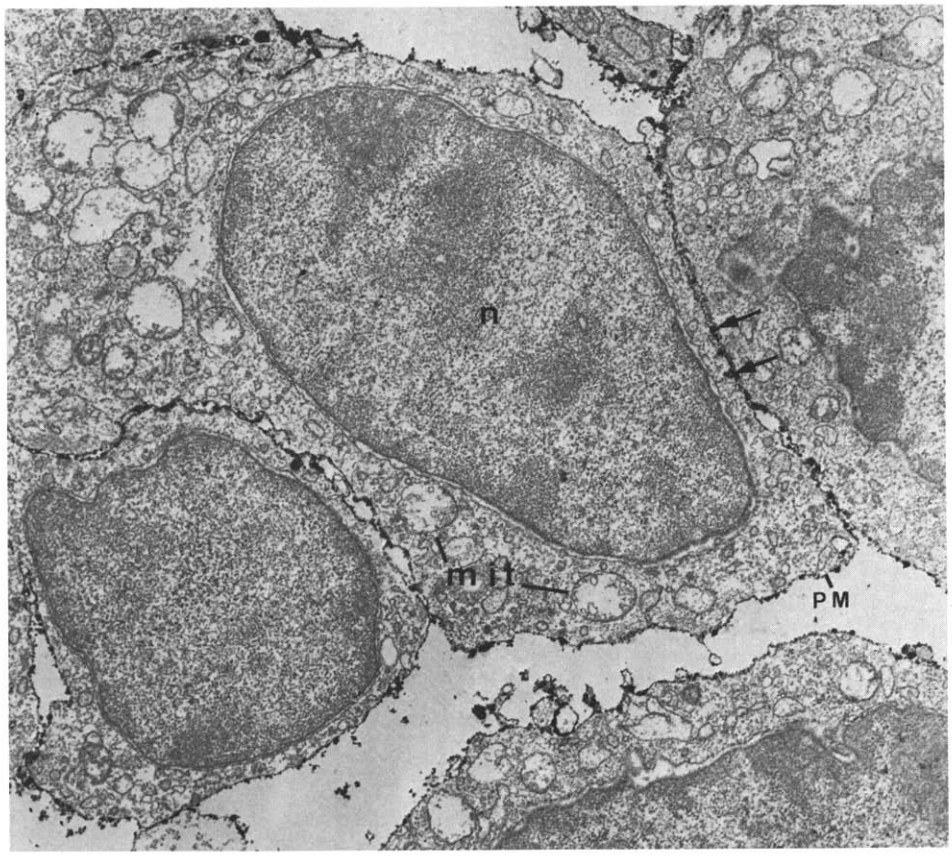

Fig. 2. Cytochemical demonstration of 5′-nucleotidase activity in murine ependymoblastoma. The tissue was fixed for 90 min in 4% paraformaldehyde and incubated for 60 min in the complete medium as described in materials and methods. The section was stained with uranyl acetate and lead citrate. n, nucleus; mit, mitochondria; PM, cell membrane. The lead precipitate (arrows) is restricted to the cell plasma membrane (×11 000).

TABLE I

DISTRIBUTION OF PROTEIN, DNA AND ENZYMES IN SUBCELLULAR FRACTIONS OF MOUSE EPENDYMOBLASTOMA

Subcellular fractionation was performed as described in Materials and Methods. Specific activity is expressed in nmol substrate hydrolysed/mg protein per min except for succinate dehydrogenase for which it is expressed in nmol tetrazolium violet reduced/mg protein per min. Enrichment refers to the ratio of the specific activity of the fraction to that in the whole homogenate. The results presented are those of a type experiment.

	Whole homogenate	Pellet P1			Pellet P2			Supernatant S2			Recovery (%)
	Specific activity	Yield (%)	Specific activity	Enrich-ment	Yield (%)	Specific activity	Enrich-ment	Yield (%)	Specific activity	Enrich-ment	
Protein	—	7.0	—	—	15.3	—	—	55.5	—	—	77.8
5′-Nucleotidase	2.84	18.0	7.50	2.6	62.2	11.58	4.1	0	0	0	80.2
Succinate dehydrogenase	3.64	8.6	4.17	1.2	64.7	15.81	4.3	0	0	0	73.3
β-Glucuronidase	1.36	5.5	1.25	0.9	24.9	2.21	1.6	76.3	1.85	1.4	106.7
Arylesterase	40.7	6.8	39.4	1.0	17.8	47.4	1.2	65.2	47.8	1.2	89.8
DNA	—	76.2	—	—	0	—	—	0	—	—	76.2

TABLE II

COMPOSITION OF PLASMA MEMBRANE FRACTION

Membrane isolation was performed as described in Materials and Methods. Enrichment is defined as the ratio of specific activity or concentration in the membrane fraction to that measured in whole homogenate. The values reported for protein and 5′-Nucleotidase are the mean of 6 experiments ± standard deviation. All other figures come from one type-experiment.

	Protein	Enzymes (nmol/mg protein per min)				Lipids μg/mg protein		
		5′-Nulceo-tidase	Phospho-diesterase I	β-Glucuron-idase	Arylesterase	Free cholesterol	Esterified cholesterol	Phospholipids
Specific activity or concentration	—	49.3 ± 13.2	25.2	4.3	65.5	166.0	27.7	681.7
Enrichment	—	15.4 ± 2.3	17.0	2.2	1.3	13.2	5.4	8.1
Yield (%)	0.46 ± 0.05	7.08 ± 1.17	7.30	1.04	0.61	6.6	2.7	3.2

not appear to affect 5'-nucleotidase activity in either the cytochemical or biochemical assays. Therefore, 5'-nucleotidase was used as a plasma membrane marker in fractionation experiments.

Preparation of plasma membrane fraction

The successive steps used in the plasma membrane preparation are summarized in Table I. The P_1-pellet, obtained at a low speed centrifugation, contained 76.2% DNA and 18% of 5'-nucleotidase activity, which were lost in this pellet.

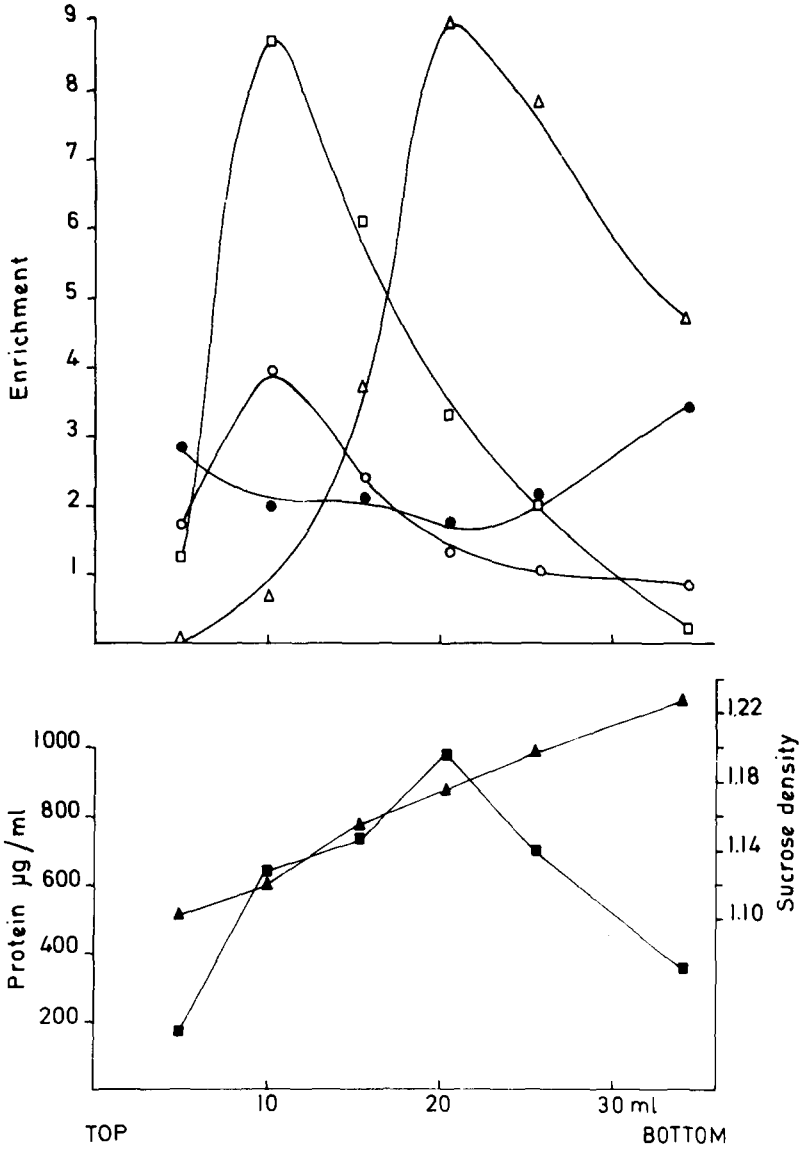

Fig. 3. Distribution of 5'-nucleotidase (□), succinate dehydrogenase (△), β-glucuronidase (○), arylesterase (●) activities, and of protein (■) after a 64 000 × g centrifugation of the P_2 pellet for 120 min in a isopycnic sucrose gradient. The enrichment refers to the ratio of specific activity in the fraction to that in the whole homogenate. The sucrose density is represented by the symbol ▲.

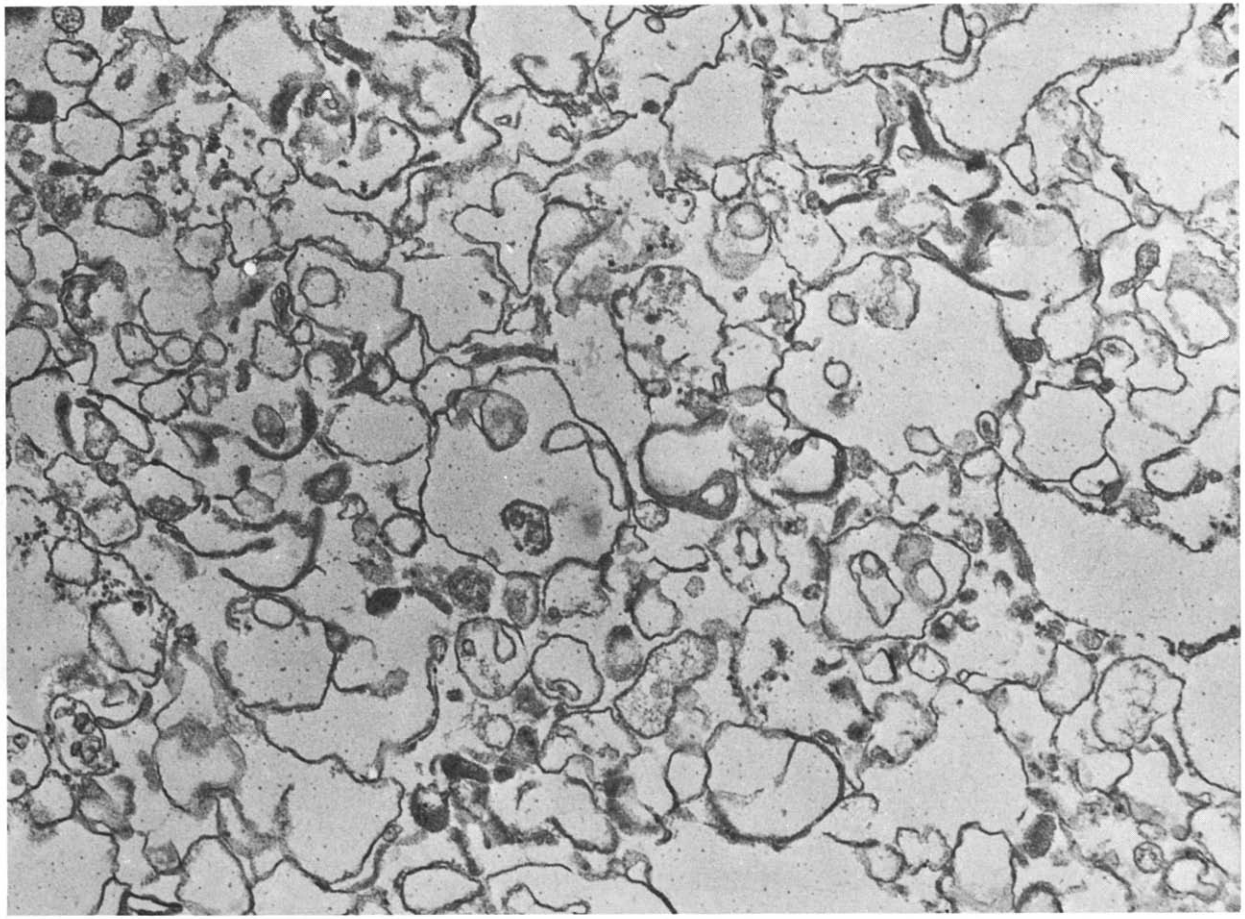

Fig. 4. Electron micrograph of the plasma membrane fraction prepared as described in materials and methods. The pellet was fixed for 90 min in 2.5% glutaraldehyde. The section was stained with uranyl acetate and lead citrate (×41 400).

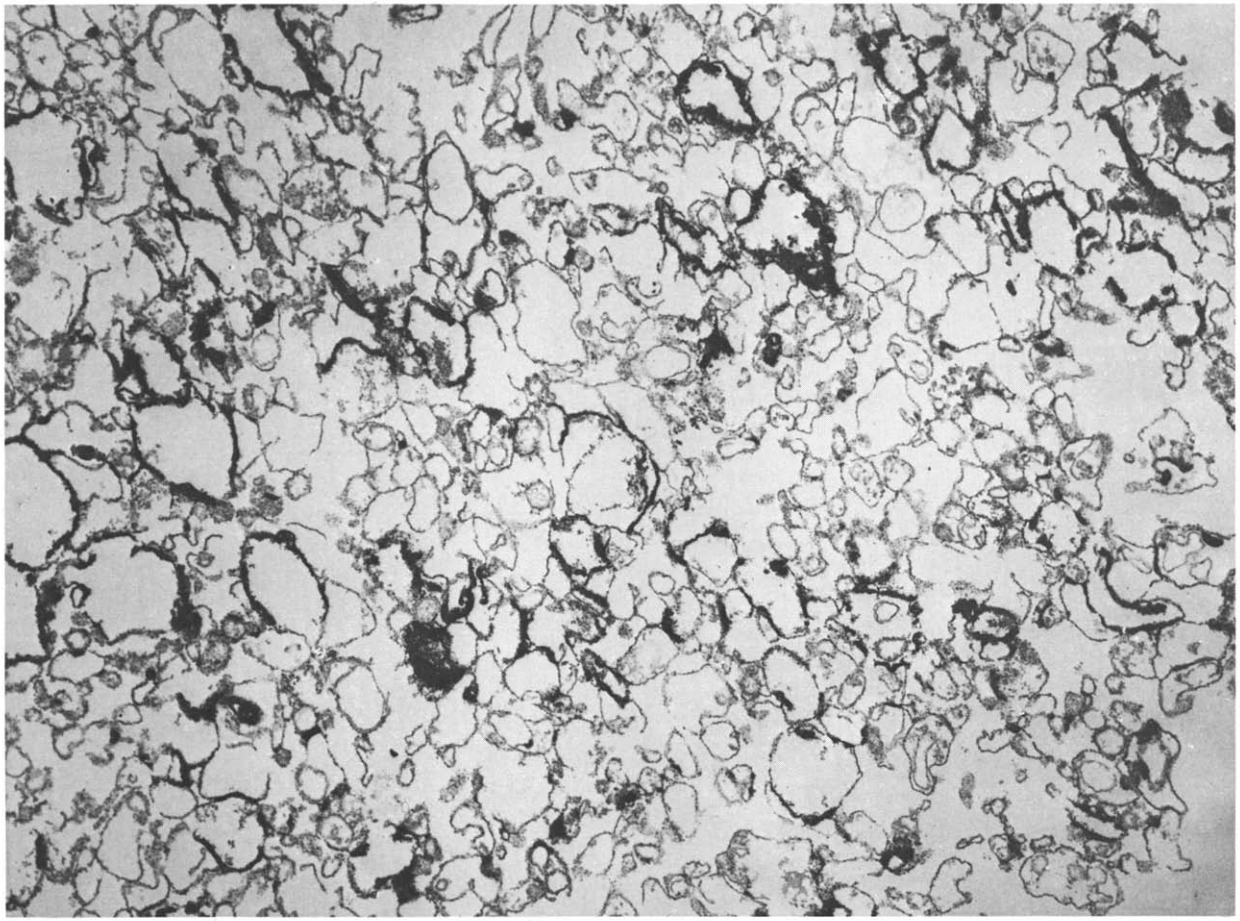

Fig. 5. Cytochemical demonstration of 5′-nucleotidase in the final plasma membrane fraction. The fraction was treated as described in Fig. 1. Heavy lead precipitate appears on many vesicles (×20 700).

TABLE III

SUBCELLULAR DISTRIBUTION OF [^{3}H]AMPHOTERICIN B

Subcellular fractionation was performed as described in Materials and Methods. Enrichment refers to the ratio of the radioactivity of the fraction to that of the whole homogenate. Except for membrane fraction, results are given for two separate experiments. Each mouse received 100 μCi of [^{3}H]Amphotericin B 10 h before sacrifice.

	Yield (%)		Radioactivity (dpm/mg protein)		Enrichment	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Whole homogenate	100	100	9 362	7 780	1	1
Pellet P1	6.2	9.4	8 217	6 544	0.9	0.8
Pellet P2	14.7	13.0	8 985	7 833	1.0	1.0
Supernatant S2	73.2	65.1	12 348	10 735	1.3	1.4
Recovery	94.1	87.5	—	—	—	—
Membrane fraction	0.7		13 260		1.4	

The P2 fraction contained 62.2% of 5′-nucleotidase and 64.7% of succinate dehydrogenase, a mitochondrial marker. The enrichment of both enzyme activities was approximately 4-fold. No 5′-nucleotidase was found in the S2 supernatant which was discarded.

Subcellular organelles from fraction P2 containing 5′-nucleotidase activity were separated from those containing succinate dehydrogenase activity by sedimentation in a continuous sucrose gradient (Fig. 3). The peak of 5′-nucleotidase activity was found at density 1.13 and that of succinate dehydrogenase activity at density 1.17. The fraction recovered at density 1.13 was characterized by a 9-fold enrichment in 5′-nucleotidase activity and a 4-fold enrichment in β-glucuronidase activity, requiring a further purification of this material by flotation in a discontinuous sucrose gradient to obtain the final fraction.

As shown in Table II, the final plasma membrane preparation was characterized by a 15.4-fold enrichment in 5′-nucleotidase activity and a 17-fold enrichment in the activity of phosphodiesterase I, another plasma membrane marker. The specific activity of β-glucuronidase is still enhanced 2.2-fold, but no succinate dehydrogenase activity could be demonstrated in this final preparation. The analysis of the lipids reveals that free and esterified cholesterol, and phospholipids were enriched respectively 13.2, 5.4 and 8.1-fold (Table II).

Electron microscopy of the plasma membrane fraction (Fig. 4) shows vesicles and occasional small fragments of rough endoplasmic reticulum. The activity of 5′-nucleotidase is cytochemically demonstrable on many membranes of this subcellular fraction: heavy lead deposits are seen on some vesicles while a fine granular precipitate appears on most other vesicles (Fig. 5).

Distribution of [^{3}H]amphotericin B

Table III shows that most of the radioactivity measured after injection of [^{3}H]amphotericin B was found in the supernatant S2. However, when the results were expressed per mg of protein, the radioactivity appeared to be similar in the different subcellular fractions. In particular, [^{3}H]amphotericin B was

only enriched 1.4-fold in the plasma membrane preparation, as compared to the whole homogenate.

Discussion

The method used for the homogenization of mouse ependymoblastoma is similar to the procedure developed by Neville for rat liver [16] and modified by Ray [17]. However, the tissue fractionation scheme followed in this work differs from that used by these authors who isolated the plasma membrane fraction from a nuclear pellet. In the procedure we used, the plasma membranes were collected in a pellet enriched in mitochondria, sedimenting at 16 000 $\times g$. Another feature of this method is the combination of isopycnic sucrose sedimentation and flotation to eliminate the contaminating organelles, namely mitochondria and lysosomes.

The reproducibility of the method is attested by the standard deviation, which is lower than 15% of the mean value of the enrichment of the final plasma membrane fraction in 5′-nucleotidase activity.

5′-Nucleotidase is the most commonly used plasma membrane marker. However, its subcellular distribution may vary from one tissue to another. For instance, in rat liver, 5′-nucleotidase is mainly located in plasma membrane, but is also present in endoplasmic reticulum [18] and Golgi apparatus [19]. On the other hand, the cytochemical studies of Misra et al. [15] have shown that, in rat lymphocytes, 5′-nucleotidase is restricted to the plasma membrane. Using the same method as Misra, we have shown that, in mouse ependymoblastoma, 5′-nucleotidase activity could only be demonstrated in plasma membranes. Therefore, it was used as a plasma membrane marker in the purification of this subcellular structure.

The final preparation of plasma membranes is enriched 15-fold in 5′-nucleotidase activity. This enrichment of the final preparation in membranes containing 5′-nucleotidase was further confirmed by electron micrograph examination.

The plasma membrane fraction was characterized by a 17-fold enrichment in phosphodiesterase I activity. This enzyme, considered by Touster [9] to be a specific component of the rat liver plasma membrane, seems to be a suitable plasma membrane marker also in mouse ependymoblastoma. The contamination of the final plasma membrane preparation by other subcellular organelles such as lysosomes, endoplasmic reticulum and mitochondria was assessed by the determination of β-glucuronidase, arylesterase and succinate dehydrogenase. The degree of contamination was acceptable, since the specific activity of β-glucuronidase was increased twice and that of arylesterase comporable to that of the whole homogenate. Succinate dehydrogenase was not detectable in the final plasma membrane fraction. Glucose-6-phosphatase is not present in mouse ependymoblastoma and was replaced by arylesterase as a marker of endoplasmic reticulum. This enzyme has been shown to be associated with endoplasmic reticulum in the rat liver [20].

The plasma membrane fraction of mouse ependymoblastoma has a phospholipid/cholesterol molar ratio of 0.6, which falls in the range of values determined for the plasma membranes of other tissues [21].

Amphotericin B is a polyene antibiotic used against fungal infections. The cytotoxic effect of the polyenes is attributed to an increase of cell permeability due to their binding to sterols of the plasma membrane [22]. The ability of amphotericin B to enhance the plasma membrane permeability [23] was also postulated to explain the potentiation of other drugs by this polyene [24–27].

In view of the results presented in this paper, it is unlikely that a binding occurs between amphotericin B and the cholesterol of the ependymoblastoma plasma membrane. The amount of [^{3}H]amphotericin B per mg of protein is roughly the same in the plasma membrane fraction as in the whole homogenate, showing that the drug has no particular affinity for the plasma membrane. In this subcellular fraction, the molar ratio of amphotericin B to total cholesterol, computed from out data, appears to be very low: 1 : 16 000.

This finding is consistent with our previous results showing that the potentiation of the therapeutic effect of a nitrosourea, the 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, by amphotericin B is probably not due to an increased uptake of the nitrosourea by the ependymoblastoma [4]. Thus, the potentiation by amphotericin B of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea cannot be attributed to an enhancing effect of the polyene on membrane permeability, as least in ependymoblastoma. In the treatment of AKR leukemia by amphotericin B and a nitrosourea, Medoff suggests that amphotericin B acts as a booster of the immune response against the tumor [28]. Indeed, it has been demonstrated recently that amphotericin B has a protective effect against spontaneous and transplantable murine tumors [29].

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