Biochimica et Biophysica Acta, 345 (1974) 180–197

BBA 76608

ON THE SIDEDNESS OF PLASMA MEMBRANE ENZYMES

EBERHARD G. TRAMS and CARL J. LAUTER

Laboratory of Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received January 4th, 1974)

SUMMARY

1. The objective of this investigation was to determine if certain marker enzymes were associated with either the exterior or interior aspect of the plasma membrane.

2. Experimentally, the problem was approached by comparing enzymatic activity of intact cells with that of disrupted cells. Because isolated cells from dispersed guinea pig liver were found to be of limited value only, the investigation was pursued in various cultured cell lines. Permeability of whole cells to substrate was excluded as a possible source of error.

3. Most of the activities of non-specific Mg^{2+}-ATPase and 5′-nucleotidase of several cell lines (i.e. HeLa; KB; human, mouse and guinea pig hepatocyte, mouse neuroblastoma) was the expression of ecto-enzymes. Phosphodiesterase I, *p*-nitrophenylphosphatase and leucyl-β-naphthylamidase also may occur preferentially as ecto-enzymes, though fewer cell lines were tested.

4. Adenylate cyclase was clearly associated with the interior aspect of the plasma membrane. Nucleotidepyrophosphatase of isolated guinea pig hepatocytes had the characteristics of an ecto-enzyme but was associated with the inside of the plasma membrane in Chang hepatocytes and KB cells.

INTRODUCTION

We have recently reviewed the problem of how plasma membranes might be characterized by marker enzymes [1] and we concluded that unique marker molecules have not been identified. The physiologic significance of many of the enzymes associated with plasma membranes is still enigmatic. Of the so called "characteristic plasma membrane enzymes" only (Na^+, K^+)-ATPase and adenylate cyclase clearly fit into our current concepts of plasma membrane function.

ATPases, 5^1-nucleotidases, phosphodiesterases, amidases and others have been reported as membrane constituents, and as markedly enriched there, with such frequency, that an inquiry into the reasons for their presence might uncover significant features

Abbreviation: EGTA, ethyleneglycol-bis(aminoethylether)-*N*,*N*′-tetraacetic acid; AMP-PNP, adenylylimide- 5′diphosphate.

of membrane biology. It seemed to us that it would be appropriate to ask first a rather simple question: Of the enzymes associated with the plasma membrane, which participate in reactions occurring on the inside of the cell and which serve a catalytic function on its outside? We have referred elsewhere to this problem as a question of sidedness of plasma membrane enzymes [2]. This is a rather awkward noun, but we believe that it states the proposition in a brief and understandable manner.

Our experimental approach to the problem of marker enzyme sidedness was as follows: When isolated intact cells were suspended in an isotonic medium containing a substrate, presumably only the outer aspect of the plasma membrane interacted with the substrate. An assay for metabolic product(s) then should reflect the activity of an ecto-enzyme only. If, however, the cells were disrupted and cytoplasmic content dispersed, barring significant sealed vesicle formation, the substrate would be exposed to the action of enzyme(s) on both sides of the plasma membrane. Ideally, one may presume that in the disrupted cell, total measured activity presented the sum of the catalytic activities of both sides, assuming that the enzyme indeed catalyzed the same reaction on both sides. If the enzyme were exclusively an ecto-enzyme, however, no increments in activity should be observed when the intact cell is compared with the disrupted cell. Intact cells, on the other hand, should assay for zero activity if the enzyme were present and acting exclusively on the inside.

In addition, the following two conditions preferably should prevail: in the case of intact (whole) cells, the substrate should remain confined to the incubation medium and not permeate the plasma membrane; in the disrupted cell, the substrate should be accessible to both sides of the plasma membrane. A desirable feature would have existed if at least one of the reaction products accumulated instead of being subject to further metabolism. As it turned out, this was rarely the case and in some instances we had to contend with qualitative results.

Our methods and reaction conditions were diverse and we have, for the sake of continuity, included some experimental details in Results.

MATERIALS AND METHODS

Cell cultures

Experiments were performed with HeLa Cells (Gey), human carcinoma of cervix (CCL 2) ("Certified Cell Line" cell repository number assigned by A.T.C.C. catalogued in ref. 3); KB cells (Eagle), human carcinoma of nasopharynx (CCL 17) [3]; HEp-2 cells (Toolan); human epidermoid carcinoma of larynx (CCL 23) [3]; L-929 cells (Earle), mouse fibroblast (CCL 1) [3]; mouse neuroblastoma clone N-18, C1300 cell line [4], obtained from Microbiological Associates, Bethesda, Md., human liver cells (Chang) (CCL 13) [3] obtained from Grand Island Biological Co., Grand Island, N.Y., and mouse hepatocytes, cell line ML-311, obtained as generous gift from Dr H. Coon, Laboratory of Cell Biology, National Cancer Institute.

Cell culture media and reagents

Dulbecco's modified Eagles medium [5, 6], Dulbecco's phosphate balanced saline [7], minimum essential medium (Eagle) [8, 6] with Earle's balanced salts solution [9] or with Hanks balanced salts solution, and NCTC-109 [10, 6] were obtained from Microbiological Associates. Hanks balanced salts solution [11] and

nutrient mixture F-12 (ref. 12) was obtained from GIBCO. A phosphate free buffer was used for incubating cells when the liberated P_i was to be measured colorimetrically in the incubation medium. The phosphate-free buffer contained: NaCl, 100 mM; KCl, 20 mM; $MgCl_2 \cdot 6H_2O$, 4 mM; $CaCl_2$, 2 mM; $NaHCO_3$, 10 mM; glucose, 5 mM; and Tris–HCl, pH 7.4, 15 mM.

Tris–[^{32}P]ATP was synthesized [13] (courtesy of Mr G. J. Koval, this laboratory) from carrier-free ^{32}P obtained from International Chemical and Nuclear Corp. (ICN), Irvine, Calif. [8-^{14}C]Adenosine 5′-monophosphate and [^{32}P]AMP-PNP was also obtained from ICN. MN-300 cellulose thin-layer chromatography plates were obtained from Analtech, Inc., Newark, Del. Aquasol liquid scintillation counter cocktail was obtained from New England Nuclear, Boston, Mass. All other chemicals were reagent grade and deionized water was employed for making solutions.

Preparation of cell cultures

Prior to use, cell suspensions were centrifuged at 4 °C for 1–4 min at 500–1000 rev./min (50–200 × *g*) and the supernatant was aspirated from the sedimented cells. The cells were pre-incubated at 37 °C for 60–120 min with appropriate culture medium, washed once with medium, and then suspended in the incubation medium required for the experimental procedures. Cell counts were made with an improved Neubauer Levy-Hausser corpuscle counting chamber (C. A. Hausser, Philadelphia, Pa.).

Cell lines grown as monolayers on Falcon plastic culture flasks were maintained in 10–20 ml of culture medium and assayed in 3–5 ml of final incubation medium.

Enzyme assays and protein content

5′-Nucleotidase (EC 3.1.3.5) was assayed mostly as described previously [14]. Standard incubations were 0.20 ml phosphate-free buffer, pH 7.4, containing 2.5 mM 5′-AMP. The reaction was stopped by addition of 0.20 ml of 10% trichloroacetic acid. After centrifugation, the liberated P_i was measured by the method of Ames and Dubin [15].

ATPase activity (EC 3.6.1.3) was assayed by a modified radioisotopic method of Weil-Malherbe and Green [16]. Incubations were in 1.1 ml of phosphate-free buffer, pH 7.4, adjusted to final concentrations of 5 mM $MgCl_2$, 40 mM KCl, 4 mM ethyleneglycol-bis(aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), and 15 mM [γ-^{32}P]ATP (about 1500 cpm/nmole).

Adenyl cyclase was assayed essentially as described by Krishna et al. [17]. Incubations were in Dulbecco's phosphate-buffered saline without fetal calf serum, pH 7.4, adjusted to final concentrations of 5 mM $MgCl_2$, 12.5 mM KF, 2 mM EDTA-Na salt, 10 mM theophylline, and from 0.5-7 μM [^{32}P]AMP-PNP (spec. act. 5–13 Ci/mmole) as substrate. After incubation but prior to the boiling step, 0.50 μmole of 3′,5′-cyclic AMP was added to each sample. A 1.0-ml aliquot was taken from the first barium precipitation step, lyophilized to dryness, then solubilized in 50 μl of water and applied to a MN-300 cellulose thin-layer chromatography plate. The plate was developed in *n*-propanol–14 M NH_4OH–water (60 : 35 : 5, v/v/v). The 3′,5′-cyclic AMP spot was scraped off and eluted with 1.0 ml water, absorbance was measured at 260 nm to determine recovery and an aliquot was counted in a liquid

scintillation spectrometer. The total labelled cyclic AMP formed was calculated from the recovery of added carrier cyclic AMP.

Dinucleotide pyrophosphatase (EC 3.6.1.9) was measured as described previously [18]. Incubations were in 1.0 ml of phosphate-free buffer, pH 7.4, adjusted to final concentrations of 10 mM $MgCl_2$, 4 mM EGTA, and 2 mM NADH. Aliquots of 0.10 ml were placed into cuvettes containing 0.90 ml of the pyruvate-lactate dehydrogenase system and the absorbance determined at 366 or 340 nm and at 410 nm to correct for turbidity, where necessary.

Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described elsewhere [14]. Incubations were in 0.20 ml of phosphate-free buffer, pH 7.4, with 8 mM EDTA, 2 mM KF, and 20–40 mM glucose 6-phosphate added. The reaction was terminated with 0.20 ml of 10% trichloroacetic acid, centrifuged, and the released P_i was measured in the supernatant by the method of Ames and Dubin [15].

The *p*-nitrophenylphosphatase (EC 3.1.3.1) was measured as previously described [14]. Incubations were in 0.90 ml phosphate-free buffer, pH 7.4, using 0.55 mM Tris–*p*-nitrophenylphosphate as substrate. The liberated *p*-nitrophenol was measured colorimetrically.

Phosphodiesterase (EC 3.1.4.1) was assayed in the same manner as *p*-nitrophenylphosphatase, except that 0.55 mM *p*-nitrophenylphosphothymidine was used as the substrate and 0.5 mM $CoCl_2$ was added to the incubation medium.

Proteins were determined by the method of Lowry et al. [19], after solubilizing the cells by addition of an equal volume of 5% sodium desoxycholate.

RESULTS

Studies on isolated guinea pig hepatocytes

Initially, we compared plasma membrane marker enzyme activity of suspensions of whole isolated liver cells with that of hepatocyte homogenates. Guinea pig liver was dispersed into individual cells by perfusion with collagenase and hyaluronidase as described previously [48]. Viability and permeability of the isolated cells was tested with trypan blue and also by assays for lactate dehydrogenase (EC 1.1.1.27) and glutamate–pyruvate transaminase (EC 2.6.1.2) in the suspension medium. By these criteria, membrane permeability was unaltered [48]. Averaged results of enzyme assays on several such preparations are shown in Table I. The differences between intact cells and homogenates were rather small. This suggested that considerable, if not most, activity of these enzymes was the result of catalysis which had occurred on the exterior aspect of the cell membrane. We were not satisfied, however, that the plasma membranes had constituted an adequate permeability barrier to the substrates. During further investigations, we subsequently could demonstrate (by methods discussed below) that the hepatocytes obtained by this method were permeable to 5′-AMP and to [^{14}C]inulin. Therefore we abandoned further experimentation with cells which had been derived from dispersed organs and we resorted to the use of various cell types which had been propagated in tissue culture.

Tissue culture experiments

Some general precautions had to be observed in the use of cultured cells. Several cell lines were usually harvested by mild trypsinization which could have re-

TABLE I

PLASMA MEMBRANE ENZYME ACTIVITY IN ISOLATED INTACT AND DISRUPTED GUINEA PIG HEPATOCYTES

Isolated liver cells prepared as described in the text. Incubation media were at pH 7.4, either isotonic saline or saline plus Tris buffer or Hanks balanced salt solution. Incubation time was from 0.25 to 0.5 h at 37 °C. Values are averaged from several individual experiments, numbers shown in parentheses.

Enzyme	Substrate	Co-factors	Activity in μmoles substrate metabolized/mg protein/h	
			Intact cells	Disrupted cells
5'-Nucleotidase	5 mM 5'-AMP	10 mM Mg^{2+}	0.385 (10)	0.431 (10)
Nucleotide-pyrophosphatase	10 mM NADH	5 mM K^+ 50 μM Co^{2+}	0.026 (4)	0.027 (4)
p-Nitrophenyl-phosphatase	0.55 mM Tris-*p*-nitrophenyl-phosphate	55 mM K^+ 11 mM Mg^{2+}	0.083 (6)	0.110 (6)
Leucyl-β-naphthyl-amidase	0.1 mM leucyl-β-naphthylamine	—	0.91 (8)	0.92 (3)

sulted in permeability changes. Therefore all cells were routinely rinsed in their specified growth medium and then preincubated in calf serum and antibiotic free media for 60–120 min at 37 °C.

Studies on substrate permeation into intact cells

Following are two examples of the method which was used to assess the degree of permeability of the cell cultures to various substrates: (1) If [γ-^{32}P]ATP had to be translocated across the plasma membrane before it could be hydrolyzed it would follow that the specific activity of intracellular orthophosphate ($^{32}P_i$) would not

TABLE II

SPECIFIC ACTIVITY OF INTRACELLULAR AND MEDIUM $^{32}P_i$ AFTER INCUBATION OF INTACT HeLa CELLS WITH [γ-^{32}P]ATP

$3.75 \cdot 10^6$ HeLa cells incubated in Dulbecco's modified Eagle's medium with 2.5 mM [^{32}P]ATP ($9.1 \cdot 10^5$ cpm/μmole). Cells sedimented, supernatant removed and each fraction adjusted to 0.3 ml and 0.2 M $HClO_4$. After addition of ammonium molybdate reagent [15] and color development, the molybdate complex was extracted with isobutanol and the absorbance and radioactivity determined as described in Methods.

	Specific radioactivity (cpm × 10^{-5}/μmole P_i)	
Incubation time (min):	10	20
ATP hydrolyzed (μmoles):	0.27	0.37
Medium P_i	7.53	8.38
Intracellular P_i	2.89	2.81

only increase rapidly but also exceed that of the extracellular (medium) $^{32}P_i$ at all times. Conversely, the specific radioactivity of extracellular $^{32}P_i$ should be higher than that of the intracellular $^{32}P_i$ if [^{32}P]ATP hydrolysis were principally due to the action of an ecto-enzyme. For the experiment summarized in Table II, batches of 10^6 HeLa cells each were incubated with [γ-^{32}P]ATP in a small volume of medium in which the phosphate concentration had been adjusted to approximate that of HeLa cells (about 1 mM) in order to minimize $^{32}P_i$ diffusion. At the end of the incubation periods, cells and medium were separated by centrifugation and orthophosphate in the respective compartments was converted to its molybdenum complex [16]. After extraction of the complex into isobutanol, one aliquot was used for scintillation counting and one for orthophosphate determination. From these data, specific radioactivity of $^{32}P_i$ in cells and medium was calculated. The specific activity of extracellular $^{32}P_i$ exceeded that of intracellular $^{32}P_i$ several fold and we concluded that ATP was hydrolyzed by HeLa cells without permeation. The background values were due to residual medium in the packed cells and some ATP hydrolysis and $^{32}P_i$ diffusion which took place during the work-up period.

(2) An improved separation of cells and medium was attained by a modification of the preceding method for experiments where 5'-[8-^{14}C]AMP served as substrate for 5'-nucleotidase assays.

Disposable glass Pasteur capillary pipets (5.75-inch-long Kimble No. 72020) were sealed at the narrow end with epoxy cement and cut to 60 mm lengths. The capillary portion was loaded with 50 μl of isotonic (9.25%, w/v) sucrose adjusted to pH 7.4 with 0.05 mM Tris–HCl. In separate small test tubes, 10^6 HeLa cells each were incubated in Dulbecco's modified Eagle's medium containing 0.1 mM 5'-[8-^{14}C]-AMP which had been purified by thin-layer chromatography. At the end of each incubation period, the incubation mixtures were loaded onto the sucrose in the Pasteur pipets and centrifuged for 5 min at 2000 rev./min in the cold. After centrifugation, the pipets were frozen in liquid nitrogen, the sedimented cell pellet was cut off, crushed and extracted with 0.25 ml of 5% trichloroacetic acid. The medium from atop the sucrose layer was collected separately and adjusted to contain 5% trichloroacetic acid. The extracts were centrifuged and extracted with ethyl ether to remove trichloroacetic acid. Aliquots of medium and cell extract (acid soluble nucleotides) were cochromatographed with adenosine, AMP, ADP and ATP on thin-layer chromatography plates (MN-300 cellulose; developed with isopropanol–NH_4OH–water, (60 : 20 : 20, v/v/v)). After development, the plates were scanned for radioactivity in a gas-flow counter or subjected to radioautography. Fig. 1 shows one of the scans for radioactivity in acid soluble fractions from medium and cells after 20 min of incubation. The largest peak from the medium is 5'-[^{14}C]AMP and the primary nucleoside metabolites are recognizable. Since the substrate was not demonstrated inside the cell and since the primary products of metabolism accumulated in the medium only, we concluded that 5'-AMP was also hydrolyzed without permeation through the plasma membrane. In this experiment, not more than 1.5% of the isotope had accumulated inside the cell during the 20-min incubation period. Radioactive material within the cell was primarily [^{14}C]ATP, probably derived from diffused nucleosides. This experiment was repeated with KB cells where the cell volume constituted $2.5\pm0.5\%$ of the medium. After 20 min of incubation, the cells contained 0.083% of $^{32}P_i$ liberated from [γ-^{32}P]ATP or 0.46% of *p*-nitrophenol

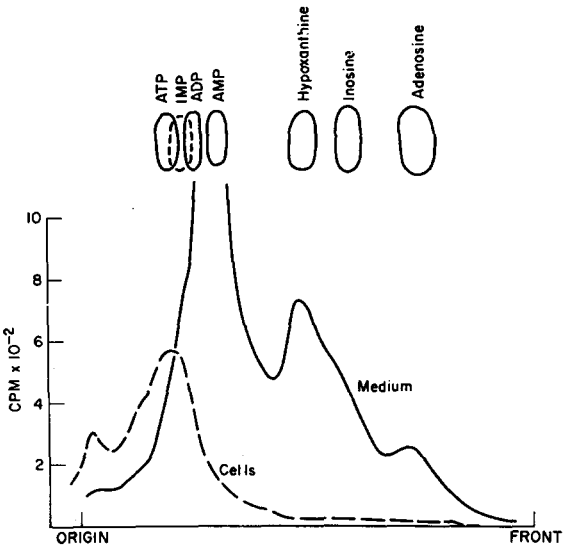

Fig. 1. Scan for radioactivity of thin-layer chromatography plate. Acid-soluble fractions of pelleted intact HeLa cells and incubation medium after 20 min of incubation with 5′-[^{14}C]AMP. Medium contains substrate and primary nucleoside metabolites; no substrate is evident inside the cells. For further details, see text.

liberated from *p*-nitrophenylphosphate. With leucyl-β-naphthylamine as substrate, about 10% of the liberated naphthylamine was associated with the pelleted cells; either the cells were permeable to leucyl-β-naphthylamine or the non-polar product was avidly taken up by the cells.

Assessment of sealed vesicle formation during cell disruption

The argument was advanced that access of substrates to the inner surfaces of plasma membrane fragments might be inhibited by the formation of sealed vesicles during cell disruption. This could lead to erroneus conclusions regarding the activity of membrane enzymes which are localized on the interior aspect of the plasma membrane. This possibility was investigated and a representative experiment is described here:

Suspensions of $2.5 \cdot 10^6$ KB cells were packed in 3/16 × 1 5/8 inch plastic centrifuge tubes at low speed (packed volume 25 mm^3). The cells were then suspended in 3 vol. of culture medium containing 1.25 mg (1.25 μCi) of [^{14}C]inulin. Two such suspensions served as whole cell controls while four others were sonicated for 10 s under our standard conditions. After maintaining the cell suspensions and sonicates for 10 and 30 min, respectively, (to allow time for resealing [20]) they were diluted to 1 ml with medium and transferred to the top of 1 × 3.5 inch centrifuge tubes which had been charged with 35 ml of cold isotonic sucrose solution buffered with 10 mM Tris–HCl, pH 7.4. The tubes were then centrifuged in an SW 27 (Spinco) rotor for 60 min at 25 000 rev./min. After centrifugation, the tubes were sliced off about 1 cm from the bottom, the pellets drained and their radioactivity measured by scintillation counting.

Displacement volume of $2.5 \cdot 10^6$ KB cells would have been equivalent to

$4.5 \cdot 10^5$ cpm (inulin entrapment for resealed complete ghosts). If the entire plasma membrane mass of the sonicated cells had been fragmented into 10^4 vesicles/cell, completely resealed and entrapped [^{14}C]inulin containing medium, about 1% of the displacement volume would have been sequestered (vesicle $r = 1000$ Å). The intact control cells carried about 0.5–0.8% of the calculated radioactivity into the high speed pellet. Over this background the pellets from sonicated cells had entrapped about $4.3 \cdot 10^3$ cpm (0.7%). Recovery of plasma membrane from sonicated cells was over 85% in the high speed pellet as determined in separate experiments with [^{3}H]concanavalin A.

Theoretically, as much as 75% of the interior aspect of the plasma membrane could have been inaccessible to substrates in disintegrated cells if the following optimal conditions had obtained: (1) complete utilization of all plasma membrane to form resealed fragments; (2) exclusion of all other membranous structures, i.e. endoplasmic reticulum [21], from contributing to sealed vesicle formation; (3) upper size of vesicles formed limited to about 1000 Å and (4) resealing to maintain original sidedness of the plasma membrane. Mason and Lee [20] observed that the resealing of membranes following short sonication periods may require several hours and homogenization generally leads to fragments considerably larger than 1000 Å (Neville, D. M., personal communication). We therefore have concluded that, although we cannot state what percentage of inside plasma membrane was excluded by vesicle formation, exclusion of substrate by this mechanism was a minor factor. Our data on adenylatecyclase sidedness strongly support this conclusion.

Ecto-ATPase and ecto-5'-nucleotidase in monolayer cultures

Mouse hepatocyte M-311 monolayer cultures in 250-ml Falcon flasks were rinsed with medium, re-equilibrated and charged with 4 ml of F12 medium containing either 0.5 mM [γ-^{32}P]ATP ($2.35 \cdot 10^7$ cpm/μmole) or 1.0 mM 5'-AMP. The flasks were incubated with gentle shaking at 37 °C and 0.2-ml aliquots of medium were removed at specified intervals for the determination of liberated P_i which was measured by scintillation counting or colorimetrically.

The results of a representative experiment (Fig. 2) indicated that hepatocyte monolayer cultures exhibited ATPase and 5'-nucleotidase activity which appeared to be associated with the plasma membrane surface.

In a similar experiment on monolayer cultures of mouse neuroblastoma cells, ATPase and 5'-nucleotidase activity were assayed in disrupted cells as well. The results (Fig. 3) not only demonstrated that ATPase and 5'-nucleotidase activity in whole cells and in disrupted cells were very similar, but also that a substantial disparity in specific enzyme activity could exist between different cell types. In the neuroblastoma, specific activity of the ATPase exceeded that of 5'-nucleotidase by a factor of about 10, while in hepatocytes, 5'-nucleotidase was about twice as active as the ATPase. The neuroblastoma ATPase activity apparently was unrelated to the (Na^+, K^+)-ATPase because in the presence of 120 mM NaCl, 10^{-3} M ouabain did not affect ATP hydrolysis in intact cells (Fig. 4). In most cell lines the differential of activity displayed by intact and disrupted cells was rather small for ATPase and 5'-nucleotidase but a substantial difference in ATPase activity was observed in the case of the mouse hepatocyte ML-311 (Fig. 5) where the ecto-enzyme activity accounted for only 25–45% of total ATPase.

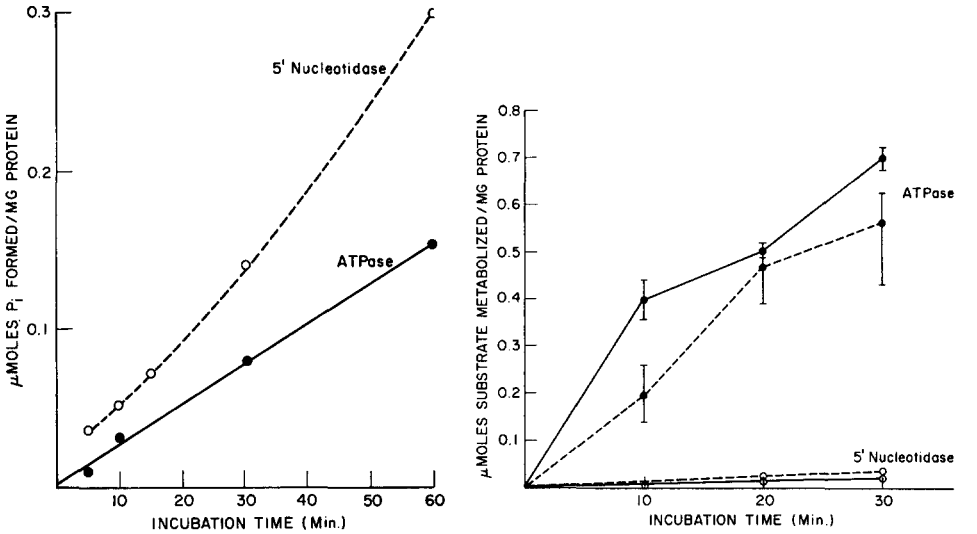

Fig. 2. 5′-Nucleotidase and ATPase activity in intact mouse hepatocytes (ML-311 monolayers). Cultures incubated with 1 mM 5′-AMP or 0.5 mM [γ-^{32}P]ATP in phosphate-free buffer and F-12 medium, respectively. Assay for liberated P_i in the medium as described in text.

Fig. 3. 5′-Nucleotidase and ATPase of intact (●- - -●) and disrupted (●–●) N-18 neuroblastoma cells. Incubated in Dulbecco's modified Eagle's medium with 0.5 mM 5′-[^{32}P]AMP or [γ-^{32}P]ATP respectively. Monolayer cultures contained an average of 1.1 mg protein per Falcon flask. Determination of liberated $^{32}P_i$ as described in text.

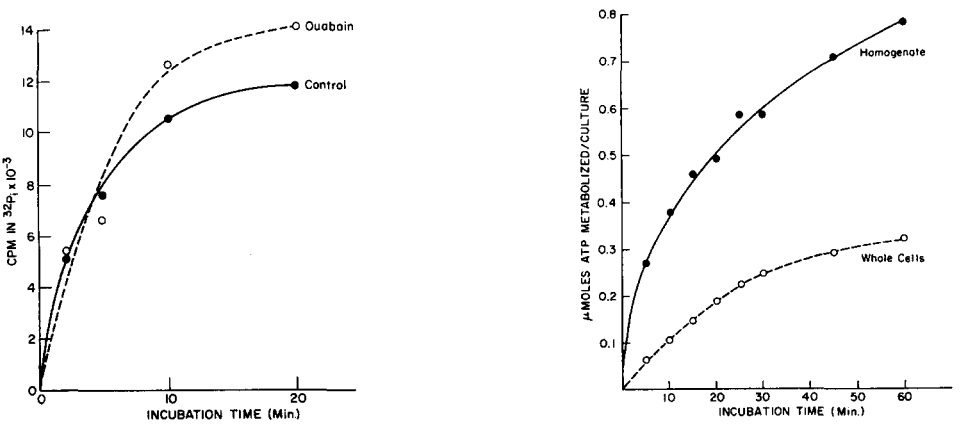

Fig. 4. Addition of 10^{-3} M ouabain to N-18 neuroblastoma monolayer cultures failed to affect ecto-ATPase activity. Substrate was 0.1 mM [γ-^{32}P]ATP; 40 mM K^+; 120 mM Na^+.

Fig. 5. ATPase activity of intact and homogenized mouse hepatocytes. ML-311 monolayer cultures incubated in F-12 medium with 0.5 mM [γ-^{32}P]ATP. Ecto-ATPase in these cells contributes only between 30–40 % of the total non-specific ATPase activity.

Comparative studies with different cell lines; Ecto-enzymes

Comparison of specific enzyme activities of different cell types or with a variety of substrates usually requires adherence to some basic enzymologic procedures. We have deviated from this tenet because of some other concerns. For instance: it is conventional procedure to assay enzyme activity at the pH optimum. We determined ATPase and 5′-nucleotidase activity in intact cells as a function of pH. ATPase displayed pH maxima at 6.5 and 8.0 and 5′-nucleotidase at 6.5 and above 8.5, respectively. In each case there was a moderate depression in the region of pH 7–7.5. We felt, however, that intact cells were least likely to incur permeability changes if they were maintained at the pH to which they had been conditioned during propagation. Therefore we elected to perform the various assays under conditions which closely resembled the pH and ionic composition of the culture media.

It is also customary to adjust substrate concentrations to correlate with the respective K_m values determined for the systems. But we observed that such adjustments just left us merely with a choice of correction factors (or errors) which had to be taken into consideration. In HeLa cells, for instance, the apparent K_m for ATP in intact cells was estimated to be 1.7 mM while in the corresponding homogenates it was found to be approximately 10 mM. Given such disparities and weighing the priorities which we had assigned to various lines of inquiry, we chose to conduct many assays without establishing optimal conditions and in most cases we have adhered to a standard protocol for intact and disrupted cells.

As might be expected, variability was a function of species, cell origin and perhaps culture conditions. Fig. 6 illustrates 5′-nucleotidase activity of four different cell types. Enzyme activity was measured in intact and disrupted cells as a function of incubation time. The data are representative of results which were obtained with

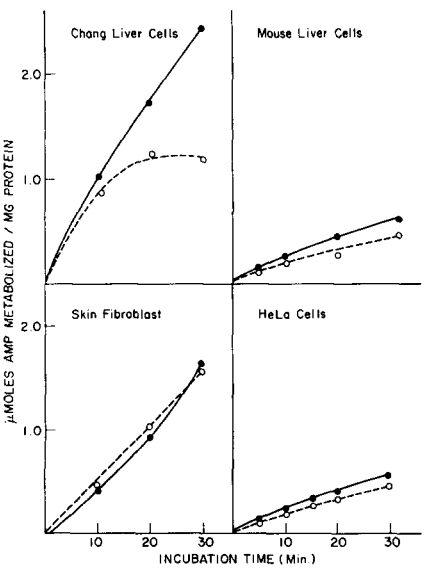

Fig. 6. 5′-Nucleotidase of intact (○- - -○) and disrupted (●—●) tissue culture cells. Incubation media were balanced salt solutions, pH 7.4, substrate 1.0 to 5.0 mM 5′-AMP. Incubation of intact cells in 250-ml Falcon flasks contained 4 ml of phosphate-free medium.

other cell type/substrate systems as well: (1) The initial rates of 5'-AMP metabolism in intact and disrupted cells were nearly identical and we believe that this supports our contention that most of the 5'-nucleotidase activity can be classified as an ecto-enzyme. (2) It confirms previous observation that two identical cell types, but derived from different species, may show significant differences in specific enzyme activity (human hepatocyte, Chang, vs. mouse hepatocyte). (3) The problem of chosing a proper incubation time is illustrated, an element which becomes particularly important when the reaction velocity is low. Accumulation of product as a function of time could be quite non-linear (i.e. Chang intact hepatocyte). After prolonged incubations, we have occasionally observed that P_i yields from ATP or 5'-AMP tended to peak and then to decline. This apparently is due to reconversion of P_i to organic phosphate (as observed elsewhere by conversion of added tracer $^{32}P_i$). (4) Occasionally, intact cells would show a slightly higher 5'-nucleotidase (or ATPase) activity than the corresponding homogenates or sonicates. This could have been due to release of an inhibitory factor during cell disruption. We tested this possibility by measuring 5'-nucleotidase and ATPase activity in admixtures of intact and disrupted cells. The resulting values were additive and therefore not compatible with the above premise.

Substrate specificity

In HeLa cells, substrate specificity of the 5'-nucleotidase was low when different 5'-nucleotides were compared (Table III). Activity varied not much more than 50% above or below the values obtained with 5'-AMP and the overall trend was similar in whole and disrupted cells. Assessment of substrate specificity in intact KB cells resulted in identical activity with the various nucleotides tested (Table III).

Table IV illustrates a similar series of experiments in which several nucleosidetriphosphates were tested for substrate specificity. Again, the enzyme did not exhibit a marked predeliction for any one substrate. This suggested that the term ATPase is a misnomer and that we were dealing with a non-specific phosphoester-

TABLE III

SUBSTRATE SPECIFICITY OF HeLa CELL AND KB CELL 5'-NUCLEOTIDASE

$2 \cdot 10^5$ HeLa cells or $3.0 \cdot 10^5$ KB cells incubated in 0.2 ml phosphate-free buffer for 15 min at 37 °C with 2.5 mM substrate. Reaction terminated with 0.2 ml 0.5 N $HClO_4$, mixture centrifuged and liberated P_i determined according to ref. 15. (Relative activity with 5'-AMP = 100.)

Substrate	HeLa		KB
	Whole cells	Disrupted Cells	Whole cells
AMP	100*	100**	100***
CMP	136	158	102
GMP	127	90	96
IMP	97	81	102
TMP	69	67	92
UMP	144	136	102
ADP	55	90	

* 0.096 μmole/h per 10^6 cells.
** 0.147 μmole/h per 10^6 cells.
*** 3.56 μmoles/h per 10^6 cells.

hydrolase, though we have adhered to the former nomenclature for the sake of continuity. Substrate specificity of the 5′-nucleotidase and ATPases were low, but it was evident that the two activities were not due to the action of a single non-

TABLE IV

SUBSTRATE SPECIFICITY OF CHANG HEPATOCYTE AND HeLa CELL ATPase IN INTACT AND DISRUPTED CELLS

Conditions same as for experiment in Table III, with 2.5 mM substrate. Liberated P_i determined essentially according to ref. 15. (Relative activity with ATP = 100.)

Substrate	Whole cells		Disrupted cells	
	HeLa	Hepatocyte	HeLa	Hepatocyte
ATP	100*	100**	100***	100§
CTP	31	64	40	47
GTP	42	87	103	80
ITP	69	96	88	87
TTP	51	115	57	73
UTP	76	95	69	63

* 0.39 μmole/h per 10^6 cells.
** 0.59 μmole/h per 10^6 cells.
*** 0.61 μmole/h per 10^6 cells.
§ 1.04 μmoles/h per 10^6 cells.

TABLE V

PLASMA MEMBRANE ENZYMES OF THREE DIFFERENT STRAINS OF CULTURED CELLS

All values are μmoles substrate metabolized per h per 10^6 cells. Methods as described in the text.

Cell type		5′-Nucleotidase	*p*-Nitrophenyl-phosphatase	Phosphodi-esterase I	ATPase
	Substrate:	2.5 mM 5′-AMP	0.55 mM *p*-nitrophe-nylphosphate	0.55 mM *p*-nitrophe-nylthymidine-5-phosphate	3 mM [γ-^{32}P]ATP
	Buffer and cofactors:	phosphate-free buffer	phosphate-free buffer	phosphate-free buffer 0.5 mM Co^{2+}	phosphate-free buffer 5 mM Mg^{2+} 40 mM K^+ 100 mM Na^+ 4 mM EGTA
HeLa	intact	0.084	0.017	0.052	0.48
	sonicated	0.136	0.024	0.072	0.60
	homogenized	0.064	0.014	0.065	0.71
KB	intact	2.19	1.70	0.012	1.66
	sonicated	2.67	2.45	0.024	1.77
	homogenized	2.55	1.84	0.017	1.88
L-929	intact	none	none	0.023	2.79
	sonicated	none	none	0.054	2.87
	homogenized	none	none	0.056	5.18

specific phosphatase. A comparative analysis of three different cell lines gave some evidence for this (Table V). For instance, ATPase activity was greatest in the L-929 mouse fibroblast, somewhat lower in KB cells and significantly lower in HeLa cells. By contrast, 5'-nucleotidase was not measurable in the L-929 cells, was greater than ATPase in KB cells and lower than ATPase in HeLa cells. Further differentiation of the two enzymic activities was obtained by studying the effects of several inhibitors. HeLa cell ATPase was completely inhibited by 10 mM EDTA while the 5'-nucleotidase showed only 70% inhibition at this concentration. In KB cells, 8 mM EDTA produced about 15% inhibition with both the ATPase and the 5'-nucleotidase. Neither enzyme was affected by the addition of 2 mM *N*-ethylmaleimide though HeLa ATPase was about 65% inhibited by ADP.

Methods of cell disruption

Table V also contains a comparison of the two different methods of cell disruption used in these experiments. It shows that homogenization and sonication gave mostly similar results, though in some instances the difference in activity between homogenized and sonicated cells was not insignificant. Some experiments were conducted on cells where adequate disruption was only achieved after the suspensions had been treated with 25 or 50 strokes of a tight fitting Dounce homogenizer. We found that cell disintegration had to be monitored microscopically in each case in order to ascertain that near complete disruption had been achieved. By contrast, treatment in an ice bath for 10 s with a Branson Ultrasonic sonifier (8–10 W output) was sufficient to disrupt more than 95% of all cell types in suspension.

Are the enzymic activities the expression of a single non-specific phosphomonoesterhydrolase?

An interesting finding in the experiments reported on Table V was the apparent absence of both 5'-nucleotidase and *p*-nitrophenylphosphatase activity in the L-929 cells. This suggested that the two activities might actually be due to the expression of a single non-specific phosphoesterhydrolase. In preliminary studies we have explored this further by using KB cells where the two enzymes have a fairly high apparent velocity. KB cell *p*-nitrophenylphosphatase was partially inhibited by both 5'-AMP and glucose 6-phosphate and the kinetics of the reactions exhibited a mixed type inhibition. Further experiments on the possible identity of ecto-5-'nucleotidase, ecto-glucose-6-phosphatase and ecto-*p*-nitrophenylphosphatase are in progress. So far, the observed low specificity for AMP and inhibition of hydrolysis by unrelated phosphomonoesters argues for the probability that the ecto-enzyme(s) are closely related to the classical alkaline phosphatase. Such an argument, however, may only apply to certain individual cell lines. Measurement of glucose-6-phosphatase in intact KB cells (Fig. 7A), for instance, yielded considerably lower values for P_i liberation than were obtained with either 5'-AMP or *p*-nitrophenylphosphatase as substrates (compare with Table V). In addition, only a fraction of the total apparent glucose-6-phosphatase of disrupted cells could be demonstrated in intact cells, indicating that less than half of the activity could be ascribed to an ecto-enzyme. Such a result was more in line with orthodox expectations since glucose-6-phosphatase is considered the classical marker enzyme for endoplasmic reticulum. Nevertheless, in view of the possibility that the outer surface of the plasma membrane

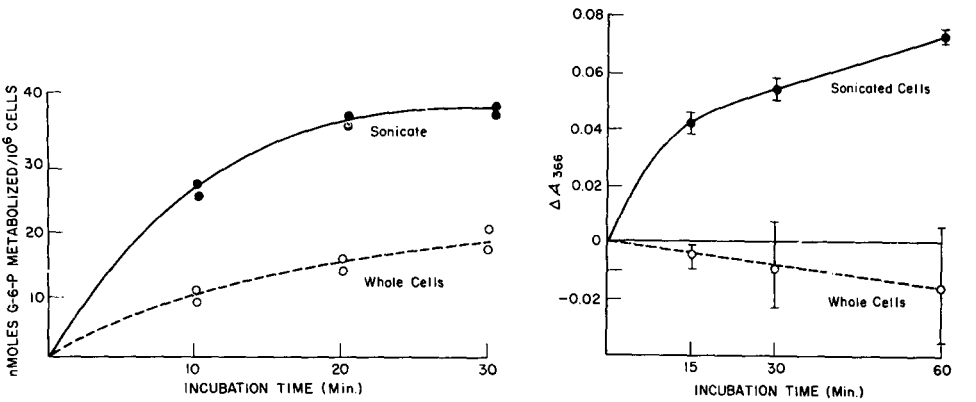

Fig. 7. A. (Left) Glucose-6-phosphatase of intact and disrupted KB cells. Incubation in phosphate-free buffer containing 10 mM glucose 6-phosphate, 1 mM KF and 4 mM Na_2-EDTA. B. (Right) Nucleotide pyrophosphatase of intact and disrupted KB cells. Incubation in phosphate-free buffer containing 2 mM NADH, 10 mM Mg^{2+}, 4 mM EGTA. The $A_{366\ nm}$ of 0.072 at the 60-min period is equivalent to 0.095 μmoles NADH per mg protein cleaved at the pyrophosphate bond.

displays the active site of a very non-specific phosphatase, the use of the term ecto-glucose-6-phosphatase may be inappropriate.

Enzymes associated with the inside of the plasma membrane

Nucleotidepyrophosphatase, when assayed under the conditions described above in intact and disrupted KB cells, did not show the characteristics of an ecto-enzyme (Fig. 7B). This finding was in agreement with our earlier observations on mammary gland plasma membranes obtained by fractionation of milk fat globules [2]. Similar results were obtained when such experiments were conducted with human hepatocyte cultures (Chang) (Table VI). Nucleotide-pyrophosphatase activity was

TABLE VI

PLASMA MEMBRANE ENZYMES OF INTACT AND DISRUPTED HUMAN HEPATOCYTES (CHANG)

Values are average of 2–4 experiments; methods as described in the text. 1 mg protein approximately $1.5 \cdot 10^6$ cells.

Enzyme	Substrate	Buffer and cofactors	Activity (μmoles/mg protein/h)	
			Intact cells	Disrupted cells
Leucyl-β-naphthyl-amidase	0.1 mM leucyl-β-naphthylamine	95 mM sodium phosphate, pH 7.4	0.46	0.79
Glucose-6-phosphatase	20 mM glucose 6-phosphate	7.0 mM histidine, pH 6.5; EDTA, 2 mM; KF, 0.5 mM	2.06	2.81
Nucleotide-pyrophosphatase	0.5 mM NADH	phosphate-free buffer	0.025	0.212

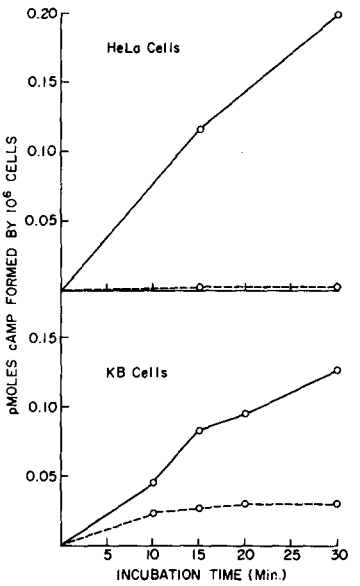

Fig. 8. Adenylate cyclase of intact (○- - -○) and disrupted (○—○) HeLa and KB cells. Incubation in Dulbecco's phosphate-buffered saline containing 3.6 and 4.0 μM [^{32}P]AMP-PNP, respectively, 5 mM Mg^{2+}, 12.5 mM KF, 2 mM EDTA and 10 mM theophylline. For details, see text.

much more evident in disrupted than in intact cells (Table VI), while at the same time both glucose-6-phosphatase and a presumptive plasma membrane marker enzyme, leucyl-β-naphthylamidase, displayed significant ecto-enzyme activity.

The one enzyme which is most clearly associated with the inside of the plasma membrane (provided that the plasma membrane is the only structure with which the enzyme is associated) was adenylcyclase (Fig. 8 and Table VII). There appeared to be some marginal activity in intact KB cells which was, however, evident only during the

TABLE VII

ADENYL CYCLASE ACTIVITY IN MOUSE NEUROBLASTOMA (N-18) AND MOUSE FIBROBLAST (L-929) INTACT AND DISRUPTED CELLS

Neuroblastoma monolayer cultures or sonicated cells incubated 20 min in Falcon flasks with 4.0 ml Dulbecco's PBS containing 0.55 μM [^{32}P]AMP-PNP and additions; average of 3 determinations each. Fibroblast intact or disrupted cell suspensions incubated in siliconized glass tubes with 1.25 ml Dulbecco's PBS containing 6.6 μM [^{32}P]AMP-PNP and additions; average of 2 determinations each. Additions and details described in Methods.

	Additions (KF)	3′,5′-cyclic [^{32}P]AMP formed (moles $\times 10^{12}$ mg protein)	
		Neuroblastoma	Fibroblast
Intact cells	None	None	0.14
Intact cells	12.5 mM	None	None
Disrupted cells	None	0.043	1.75
Disrupted cells	12.5 mM	0.180	14.3

first few minutes of incubation. We were unable to demonstrate the formation of 3′,5′-cyclic [^{32}P]AMP from [^{32}P]AMP-PNP in intact cells. The adenylcyclase characteristically was stimulated by fluoride as shown in the experiments with neuroblastoma monolayer cultures (Table VII). In all other experiments, F^- was routinely added to the incubation mixtures. Since it was not relevant to our study, we have made no attempts to explore the effects of insulin, norepinephrine, or other hormones.

We have attempted to measure succinate dehydrogenase (EC 1.3.99.1) in intact KB and HEp cells. No measurable activity was associated with the intact cells while, as expected, disintegrated cells displayed the activity of the mitochondrial marker enzyme. These observations served as additional controls for the studies reported above.

DISCUSSION

On the preceding pages we have documented that plasma membranes exhibit a transverse asymmetry in respect to the catalytic site of certain marker enzyme molecules. Data, derived from a variety of cell lines, show that 5′-nucleotidase(s) and ATPase(s) are associated with – or face – the external aspect of the plasma membrane. Leucyl-β-naphthylamidase and a non-specific phosphomonoesterhydrolase also appear as ecto-enzymes. Adenylcyclase was associated only with the internal aspect of the plasma membrane. This finding was presaged by the earlier observations of Davoren and Sutherland [22] and Rodbell et al. [23]. Nucleotidepyrophosphatase, while seemingly an ecto-enzyme in isolated guinea pig liver cells, was localized preferentially on the inside of the Chang liver cell and the KB cell.

Since these conclusions are based mostly on data derived from cultured cell lines, and since other patterns may occur in intact organs, it need hardly be emphasized that the above experiments represent a mere exploration of the question of "sidedness" of plasma membrane molecules. That such sidedness is not restricted to macromolecules may be inferred from the observations of Gordesky and Marinetti [24] who recently described an asymmetric arrangement for some phospholipids in the human erythrocyte membrane.

An apparent asymmetry in plasma membrane enzyme distribution had been suggested by a few earlier studies. W. A. Engelhardt reported the occurrence of an ATPase which seemed to be localized on the surface of nucleated erythrocytes [25] and he coined the term ecto-enzyme. A few years earlier Acs et al. [26] had observed that more than 90% of the ATPase activity of Ehrlich ascites cells seemed to be associated with the cell surface. The enzyme was fairly specific for ATP and much less activity was obtained when either hexosediphosphate, glycerophosphate or AMP were used as substrates. Novikoff [27] reported that he visualized an ecto-ATPase histochemically in rat hepatoma and subsequently Essner et al. [28] reported the presence of ATPase and 5′-nucleotidase in rat liver plasma membranes as revealed by electron microscopy. It was their contention that they had visualized the activity of ecto-enzymes and that they dealt with substrate-specific phosphatases rather than with an alkaline phosphatase. At about the same time Venkstern and Engelhardt [29] communicated further details on the distribution and properties of an ecto-adenosine-polyphosphatase; they found that the enzyme was stimulated by Mg^{2+} and inhibited by $3 \cdot 10^{-4}$ M EDTA.

That ecto-enzymes are not a peculiarity of malignant or cultured cells can be inferred from the following observations: Sells et al. [30] described an ecto-ATPase in *Amoeba proteus*, Marsh and Haugaard [31] found ATPase activity in intact cells of rat diaphragm and Cummins and Hyden [32] reported the occurrence of an ecto-ATPase in intact glia (though they were unable to demonstrate any ecto-enzyme in intact neurons). More recently Kemp [33] described the presence of an ecto-ATPase on chick embryo muscle fibroblasts.

The presence of yet another ecto-enzyme was suggested by the results of one of our control experiments: HeLa cells exposed to 5′-[^{14}C]AMP gave no indication of substrate penetration into the cells; the finding of [^{14}C]ATP within the cells and of labeled adenosine and its metabolites in the medium was consistent with metabolic pathways reported by others [34–36]. But these observations also imply that an ecto-adenosine (or AMP) deaminase existed on the HeLa cell plasma membrane.

It might also be of interest to cite two studies published by Decker's group [37, 38] on the in vivo permeability of hepatocytes to nucleotides. These investigators concluded that neither UDP-glucose nor coenzyme A permeated into the liver cell from the circulation. They did observe, however, that both substrates were rapidly metabolized. The nature of the reaction products which were recovered indicates that the catabolism of their nucleotides was mediated by an ecto-nucleotidepyrophosphatase.

Finally, it is proper to ask the question: Does our limited experimental sample allow us to draw any conclusions about the "sidedness" of plasma membrane enzymes in general? In answer to this question: We believe to have recognized a widely applicable distribution pattern for at least two plasma membrane enzymes. We predict that most eucaryotic cells will display the activity of a non-specific, Mg^{2+}-stimulated ecto-nucleosidetriphosphate-phosphoesterhydrolase (ATPase). The significance of this ecto-ATPase will be the subject of another communication. Adenylate cyclase should be confined to the interior aspect of the plasma membrane. Exceptions to this distribution pattern may only serve to prove the rule. We also postulate that the occurrence of an ecto-nucleosidemonophosphate-phosphoesterhydrolase (5′-nucleotidase) shall be common to many cell types, though exceptions will probably be more abundant than in the first case. Our data are insufficient for generalized assignments of sidedness of leucyl-β-naphthylamidase, phosphodiesterase, alkaline phosphatase and nucleotidepyrophosphatase. In the latter cases, assignment of sidedness, even if only by preference at present, fails to contribute an immediate assignment of function, though the membrane bound nucleotide pyrophosphatase activity may be an expression of multiglycosyltransferase systems which have been implicated in intercellular adhesion [39, 40]. In the case of the non-specific ATPase, some speculations about its function have been made by others. Thus, alteration of the cell surface (mediated by ATP) is a common denominator of suspected ecto-ATPase function [41, 42] and contractile actomyosin-like membrane proteins with ATPase activity may play a role in cell adhesion [43]. Translocation of molecules which participate in metabolic processes may require ATP [44–46] and the finding that ATP can be produced extra-cellularly by normal and neoplastic cells in culture [47] would seem to reinforce this line of thought.

REFERENCES

1 Solyom, A. and Trams, E. G. (1973) Enzyme 13, 329–372
2 Patton, S. and Trams, E. G. (1971) FEBS Lett. 14, 230–232
3 American Type Culture Collection (1972), Registry of Animal Cell Lines, 2nd edn (Shannon, J. E. and Macy, M. L., eds), unpaged, Rockville, Md.
4 Seeds, N. W., Gilman, A. G., Amano, T. and Nirenberg, M. W. (1970) Proc. Natl. Acad. Sci. U.S. 66, 160–167
5 Dulbecco, R. and Freeman, G. (1959) Virology 8, 306–397
6 Morton, H. J. (1970) In Vitro 6, 89–108
7 Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99, 167–182
8 Eagle, H. (1959) Science 130, 432–437
9 Earle, W. R. (1943) J. Natl. Cancer Inst. 4, 165–212
10 Evans, V. J., Bryant, J. C., Fioramonti, M. C., McQuilkin, W. T., Sanford, K. K. and Earle, W. R. (1956) Cancer Res. 16, 77–86
11 Hanks, J. H. and Wallace, R. E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196–200
12 Ham, R. G. (1965) Proc. Natl. Acad. Sci. U.S. 53, 288–293
13 Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149
14 Lauter, C. J., Solyom, A. and Trams, E. G. (1972) Biochim. Biophys. Acta 266, 511–523
15 Ames, B. N. and Dubin, D. T. (1960) J. Biol. Chem. 235, 769–775
16 Weil-Malherbe, H. and Green, R. H. (1951) Biochem. J. 49, 286–292
17 Krishna, G., Weiss, B. and Brodie, B. B. (1968) J. Pharmacol. Exp. Ther. 163, 379–385
18 Skidmore, J. and Trams, E. G. (1970) Biochim. Biophys. Acta, 219, 93–103
19 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20 Mason, W. T. and Lee, Y. F. (1973) Nat. New Biol. 244, 143–145
21 Leskes, A., Siekevitz, P. and Palade, G. E. (1971) J. Cell. Biol. 49, 288–302
22 Davoren, P. R. and Sutherland, E. W. (1963) J. Biol. Chem. 238, 3016–3023
23 Rodbell, M., Birnbaumer, L. and Pohl, S. L. (1970) J. Biol. Chem. 245, 718–722
24 Gordesky, S. E. and Marinetti, G. V. (1973) Biochem. Biophys. Res. Commun. 50, 1027–1031
25 Engelhardt, W. A. (1957) Proc. Int. Symp. Enz. Chem. (Tokyo and Kyoto) 2, 163–166
26 Acs, G., Ostrowski, W. and Straub, F. B. (1954) Acta Physiol. Acad. Sci. Hung. 6, 261–263
27 Novikoff, A. B. (1957) Cancer Res. 17, 1010–1027
28 Essner, E., Novikoff, A. B. and Masek, B. (1958) J. Biophys. Biochem. Cytol. 4, 711–716
29 Venkstern, T. V. and Engelhardt, A. V. (1957) Biokhimiya 22, 855–860
30 Sells, B. H., Six, N. and Brachet, J. (1961) Exp. Cell. Res. 22, 246–256
31 Marsh, J. B. and Haugaard, N. (1957) Biochim. Biophys. Acta, 23, 204–205
32 Cummins, J. and Hyden, H. (1962) Biochim. Biophys. Acta 60, 271–283
33 Kemp, R. B. (1972) Sci. Tools (LKB) 19, 3–5
34 Williams, A. M. and LePage, G.A. (1958) Cancer Res. 18, 548–553
35 Maguire, M. H., Lukas, M. C. and Rettie, J. F. (1972) Biochim. Biophys. Acta 262, 108–115
36 Shimizu, H., Tanaka, S. and Kodama, T. (1972) J. Neurochem. 19, 687–698
37 Bischoff, E., Liersch, M., Keppler, D. and Decker, K. (1970) Hoppe-Seylers Z. Physiol. Chem. 351, 729–736
38 Domschke, W., Liersch, M. and Decker, K. (1971) Hoppe-Seylers Z. Physiol. Chem. 352, 85–88
39 Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297
40 Roth, S. and White, D. (1972) Proc. Natl. Acad. Sci. U.S. 69, 485–489
41 Knight, V. A., Jones, M. B. and Jones, P. C. T. (1966) Nature 210, 1008–1010
42 Jones, M. B. (1966) Nature 212, 362–365
43 Jones, P. C. T. (1966) Nature 212, 365–369
44 Neifakh, S. A., Avramov, J. A., Gaitskhoki, V. S., Kazakova, T. B., Monakhov, N. K., Repin, V. S., Turovski, V. S. and Vassiletz, I. M. (1965) Biochim. Biophys. Acta 100, 329–343
45 Karjalainen, E. (1968) in Regulatory Functions of Biological Membranes (Järnefelt, J., ed.), pp. 107–115, Elsevier, Amsterdam
46 Montagnier, L. (1970) in 2me Colloque International sur les Virus Oncogènes, pp. 19–32, C.N.R.S
47 Agren, G., Ponten, J., Ronquist, G. and Westermark, B. (1971) J. Cell. Physiol. 77, 331–336
48 Solyom, A., Lauter, C. J. and Trams, E. G. (1972) Biochim. Biophys. Acta 274, 631–637