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THE NUCLEOTIDE PHOSPHOHYDROLASE ACTIVITY OF CHICK EMBRYO FIBROBLASTS

JAMES F. PERDUE

McArdle Laboratory for Cancer Research, Medical Center, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

The properties and distribution of nucleotide phosphohydrolases were examined in intact and broken preparations of chick embryo fibroblasts. These cells have a specific ADPase (EC 3.6.1.6) associated preferentially with the plasma membrane. This nucleotide diphosphate was hydrolyzed at an optimum of pH 7.8 and at a Mg^{2+} to ADP ratio of 1:1. The conditions for maximum hydrolysis of ATP by ATPase (EC 3.6.1.3) were established with respect to protein concentration, pH and mono- and divalent ion specificity and concentration.

INTRODUCTION

Energy-yielding nucleotide phosphatases have been implicated in the transport of specific ions and metabolites across the cell membrane. The $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3) is the most widely studied example of an enzyme system, incorporated within a membrane, which functions as a mediator of ion transport. This system, found in most cells[1–3], is associated with the plasma membrane as evidenced by its presence in red blood cell ghosts[4], the isolated surface membranes from Ehrlich ascites cells[5], rat liver cells[6], ventral prostate cells[7], *etc.*, and by the demonstration of substrate and ion specific enzyme reactions on the inner surface of red blood cell ghosts with electron microscopic–histochemical procedures[8].

The presence of specific nucleotide-phosphohydrolyzing enzymes within the cell membrane has provided markers for the isolation of this membrane from ascites cells[5], liver cells[6], HeLa cells[9], *etc.* A study was initiated to determine the localization and properties of phosphohydrolyzing enzymes associated with intact and broken chick embryo fibroblasts as an initial step in attempts to isolate the plasma membrane from these cells and from cells which had been converted by the oncogenic RNA sarcoma viruses[10].

The results of this study showed that there are active ADP-, CDP-, CTP-, and UTPases associated principally with the cell membrane and thus are useful as markers for the isolation of the plasma membrane[11].

MATERIALS AND METHODS

Fertilized chicken eggs were supplied by Sunnyside Hatchery of Oregon, Wisc. The following were purchased from Sigma Chemical Co., St. Louis, Mo.: the sodium salts of 5'-triphosphates of adenosine, cytidine, guanosine, inosine and uridine and the 5'-monophosphates of adenosine and inosine. The adenosine 5'-triphosphate was also obtained as the Tris salt. The following were purchased from CalBiochem, Los Angeles, Calif.: the 5'-diphosphates of adenosine, cytidine, guanosine, inosine and uridine and the 5'-monophosphates of cytidine, guanosine and uridine as the sodium salts. Glucose-6-phosphate dehydrogenase, hexokinase and $NADP^+$ were purchased from the Sigma Chemical Co.

Culturing and harvesting of cells

Primary cultures of chick fibroblasts were prepared from 13-day-old embryonated eggs by a method described by TEMIN[12] and were cultured and harvested in the log phase of growth according to a recently published procedure[11]. The cells were suspended in 0.25 M sucrose (pH 7.0), centrifuged, and washed twice by resuspension in sucrose and centrifugation.

A uniform breakage of the cells, suspended in 2.5–3.0 ml of 0.25 M sucrose, was achieved by sonication or by homogenization. Sonication was carried out in an alcohol–dry ice bath for 8 sec at a setting of 3 with a Branson Instruments, Inc. Sonifier, Model LS-75, equipped with a microprobe.

Protein determinations

Protein was determined by the procedure of LOWRY *et al.*[13] with bovine serum albumin as the standard.

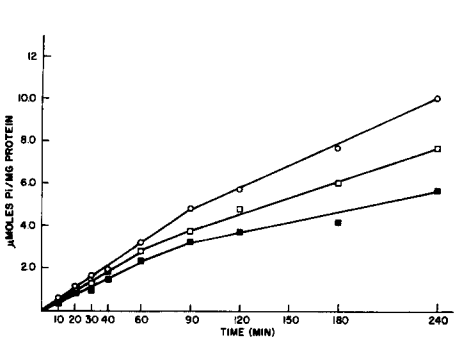
Enzyme assays

The phosphohydrolase activity of intact and broken cells was determined by incubating 25–100 μg of protein in a total volume of 1 ml containing 20 μmoles of buffer, 3 μmoles of substrate, 3 μmoles of $MgSO_4$, ions, water and sucrose to a final concentration of 250 mosM. When added, the Na^+, K^+, Ca^{2+} or Mn^{2+} were the chloride salts. The reaction mixture was incubated for 60 min at 37°. The quantity of P_i that was released from the substrate was measured by the procedure of MARTIN AND DOTY[14] as modified by LINDBERG AND ERNSTER[15], and the mean of triplicate values was expressed as μmoles P_i released per 60 min/mg protein. The specific conditions of incubation are given in the legends to the figures or in the tables.

RESULTS

Chick embryo fibroblasts which were detached from the culture dish with collagenase remained viable, as indicated by their exclusion of the vital dye, Trypan Blue. Chemical determinations of sialic acid[11] established that negligible quantities of this surface membrane component were lost from the cells by treatment with collagenase. Since one of the objectives of this study was to establish conditions of assay for those nucleotide phosphohydrolases which were found to be present on the cell surface, it was essential that the plasma membrane be undamaged.

The incubation of intact cells in an isotonic medium (pH 7.8), containing Mg^{2+} and ATP or ADP, released P_i linearly for up to 90 min (Fig. 1). The quantity



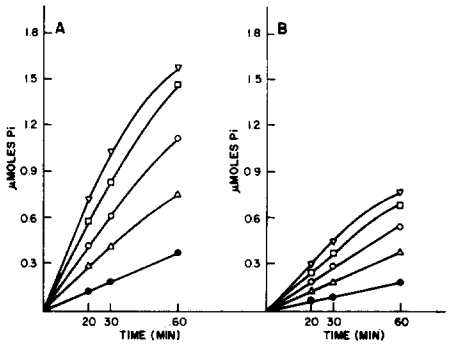


Fig. 1. The hydrolysis of ATP and ADP by intact cells with or without a hexokinase system as a function of time. Chick embryo fibroblasts were incubated at 37° in a total volume of 1 ml containing 3 µmoles $MgSO_4$, 20 µmoles Bicine buffer (pH 7.8), 120 µg protein, and sucrose to a final concentration of 250 mosM. The degree of hydrolysis of 3 µmoles of ATP (○–○), 3 µmoles ADP (□–□), and 3 µmoles ADP + 20 µmoles of glucose, 1 µmole NADP, 1 unit of hexokinase and 0.18 unit of glucose-6-phosphate dehydrogenase (■–■) was determined following varying lengths of incubation.

Fig. 2. The hydrolysis of ATP and ADP by intact cells as a function of time and protein concentration. A. Chick embryo fibroblasts were incubated at 37° for 20, 30 or 60 min in a total volume of 1 ml containing 3 µmoles ATP, 3 µmoles $MgSO_4$, 20 µmoles Bicine buffer (pH 7.8), sucrose to 250 mosM and varying concentrations of protein: 98 (●–●), 196 (△–△), 294 (○–○), 392 (□–□) and 490 µg (▽–▽). B. Chick embryo fibroblasts incubated with 3 µmoles ADP under identical conditions as in A and with varying protein concentrations: 111 (●–●), 222 (△–△), 333 (○–○), 444 (□–□) and 555 µg (▽–▽).

TABLE I

THE HYDROLYSIS OF ADP AND ATP BY INTACT CELLS IN THE PRESENCE OF IONS AND OUABAIN

Chick embryo fibroblasts were incubated at 37° for 60 min in a 1-ml volume containing 20 µmoles Bicine buffer (pH 7.8), indicated ions, ouabain, ADP or ATP, and sucrose to a final concentration of 250 mosM. The mean ± S. E. was calculated from three experiments.

Incubations conditions		*µmoles of P_i released per 60 min per mg protein*	
Ions	*µmoles*	*ADP*	*ATP*
No ions	—	0.20 ± 0.05	0.35 ± 0.05
Mg^{2+}	3	1.10 ± 0.26	1.68 ± 0.25
Mg^{2+} + Na^+	3 + 80	1.11 ± 0.26	1.75 ± 0.20
Mg^{2+} + Na^+ + K^+	3 + 80 + 10	0.97 ± 0.19	1.89 ± 0.18
Mg^{2+} + Na^+ + K^+ + ouabain	3 + 80 + 10 + 0.5	0.99 ± 0.17	1.73 ± 0.14
No ions	—	0.42 ± 0.15	0.42 ± 0.12
Mg^{2+}	1	2.59 ± 0.15	2.22 ± 0.24
	3	4.00 ± 0.30	3.93 ± 0.25
	6	3.25 ± 0.32	3.81 ± 0.21
Mn^{2+}	1	2.97 ± 0.30	2.41 ± 0.21
	3	2.71 ± 0.26	2.99 ± 0.39
	6	1.88 ± 0.25	2.40 ± 0.29
Ca^{2+}	1	3.21 ± 0.33	2.89 ± 0.28
	3	2.88 ± 0.32	3.90 ± 0.29
	6	2.44 ± 0.24	3.20 ± 0.18

of P_i released from the ATP varied from about twice to an amount almost equal to that released from ADP (Table I). The presence of an active nucleotide diphosphatase on the cell surface prompted a further study of its properties as well as those of the ATPase.

The hydrolysis of ADP and ATP was proportional to the amount of cell protein up to 300–400 μg protein/ml (Figs. 2A and 2B). The quantity of protein used for most of the determinations was about 100 μg, but on occasion up to 200 μg has been used. The enzymatic hydrolysis of both substrates was stimulated by Mg^{2+} (Table I). It was found that the optimum concentration for this divalent ion was 3 μmoles in the presence of 3 μmoles of ATP or ADP. Increasing levels of Mg^{2+} produced a slight inhibition of P_i release. The Mg^{2+}-to-substrate ratio of 1:1 which was found optimum for the hydrolysis of ADP and ATP by these cells was also found to be optimum for the hydrolysis of ATP by crab nerve membranes[2], avian salt gland homogenates[16], human erythrocytes[4], and membranes from the ventral prostate of the rat[7]. The hydrolysis of the ADP and ATP was also stimulated by Ca^{2+} and Mn^{2+} (Table I). However, the optimum divalent ion-to-substrate ratio differed between the ATPase and ADPase. The nucleotide diphosphate was hydrolyzed to the greatest extent at a ratio of 1:3, whereas the stimulated hydrolysis of ATP by Ca^{2+} and Mn^{2+} was similar to that produced by Mg^{2+}, that is, at an ion-to-substrate ratio of 1:1. The ATPase activity of the chick fibroblast in the presence of Ca^{2+} was equal to its activity with Mg^{2+}.

In the intact cell, the Mg^{2+}-ATPase was not significantly increased by the addition of $Na^+ + K^+$; the Mg^{2+}-ADPase was similarly unaffected (Table I). This failure to stimulate the hydrolysis of Mg^{2+}-ATP, an indication of the presence of the specific "ion transport" enzyme, may be explained according to proposed models for this enzyme[2,3,17] by the inability of exogenous Na^+ and ATP to penetrate the intact cells and catalyze the phosphorylation of the ion transport protein on its intracellular membrane surface. In the proposed model of transport the phosphorylated protein releases P_i during the movement of extracellular K^+ to the inside of the cell. Therefore, in order to determine the properties of the fibroblast ion transport ATPase, it was necessary to break up the cells by homogenization.

The Mg^{2+} concentration which stimulated the maximum rate of hydrolysis by fibroblast homogenates of ADP, ATP and ATP in the presence of Na^+ and K^+ remained at 3 μmoles with 3 μmoles of substrate (Fig. 3). SKOU[2] had observed that with most ion transport enzymes the most effective Mg^{2+}-to-ATP ratio increased from 1:1 to 2:1 when $Na^+ + K^+$ was added. However, AHMED AND WILLIAMS-ASHMAN[7] have also found that the Mg^{2+}-to-ATP ratio for the ion transport enzyme from the rat ventral prostate remained at 1:1 in the presence of $Na^+ + K^+$.

In whole cells, the addition of Na^+ to the reaction mixture resulted in only slight stimulation of the Mg^{2+}-ATPase even in the presence of 10 μmoles of K^+ (Table I, Fig. 4). The addition of Na^+ to cell homogenates was also without effect on the rate of ATP hydrolysis in the absence of K^+, but in the presence of 10 μmoles of K^+ there was a marked increase in the activity of the ion transport enzyme. A level of 40–80 μmoles of Na^+ was found to be the optimum concentration for the stimulated hydrolysis of Mg^{2+}-ATP.

Incubation of fibroblast homogenates with 40 or 80 μmoles of Na^+ produced a stimulated release of P_i in the presence of K^+ (Table I, Fig. 5). A concentration

of 10–20 μmoles of K^+ was found to be optimum for ATP hydrolysis, whereas higher levels of this ion resulted in some inhibition of the ATPase. In the absence of Na^+, K^+ had little effect on the ion transport enzyme in either the intact or the homogenized cells (Fig. 5).

Ouabain at a concentration of 0.05 μmole per 100 μg of cell protein inhibited the ion transport ATPase by about 60%. Increasing concentrations of the cardiac glycoside decreased this enzymatic activity still further (Fig. 6). The ouabain had only a small effect upon the Mg^{2+}-ATPase of the intact cells.

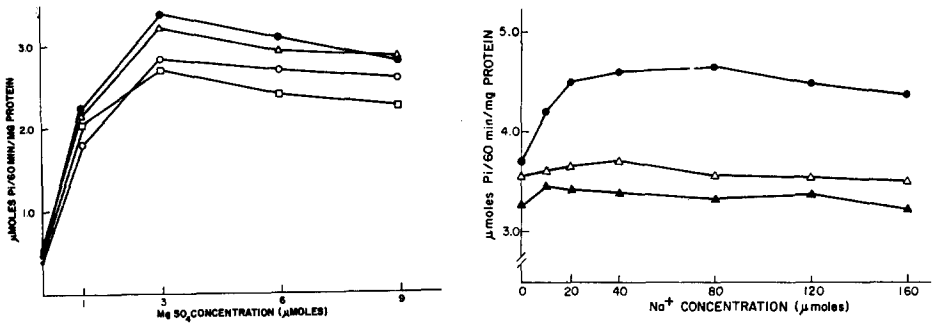


Fig. 3. The hydrolysis of ATP and ADP by cell homogenates as a function of ions and $MgSO_4$ concentration. Cell homogenates were incubated at 37° for 60 min in a volume of 1 ml containing 111 μg protein, 20 μmoles Bicine buffer (pH 7.8), and sucrose to 250 mosM. The degree of hydrolysis of ADP (□–□), ATP (○–○), ATP + 80 μmoles Na^+ (△–△) and ATP + 80 μmoles Na^+ + 10 μmoles K^+ (●–●) was determined at various $MgSO_4$ concentrations.

Fig. 4. The hydrolysis of ATP at various Na^+ concentrations by intact and homogenized fibroblasts. Intact cells were incubated at pH 7.8 with 3 μmoles $MgSO_4$, 3 μmoles ATP, 10 μmoles K^+ and 100 μg protein (▲–▲). Homogenized cells were incubated with 3 μmoles $MgSO_4$, 3 μmoles ATP, and 100 μg protein (△–△) or with 3 μmoles $MgSO_4$, 3 μmoles ATP, 10 μmoles K^+ and 100 μg protein (●–●).

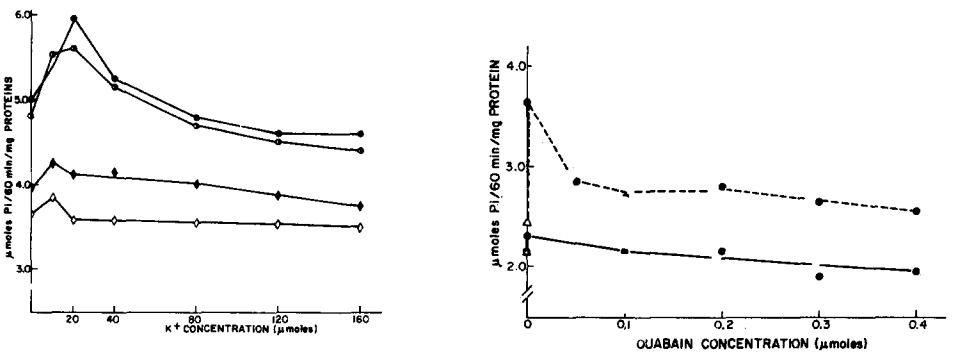


Fig. 5. The hydrolysis of ATP at various K^+ concentrations by intact and homogenized fibroblasts. Intact fibroblasts were incubated at pH 7.8 with 3 μmoles of $MgSO_4$, 3 μmoles of ATP, 100 μg protein (◇–◇) and similar conditions but including 80 μmoles Na^+ (◆–◆). Cell homogenates were incubated with Mg^{2+} and ATP and 40 μmoles of Na^+ (◑–◑) or 80 μmoles of Na^+ (●–●).

Fig. 6. The inhibition of (Na^+ + K^+)-stimulated Mg^{2+}-ATPase at various ouabain concentrations. Intact and homogenized fibroblasts were incubated at pH 7.8 in a medium containing 3 μmoles $MgSO_4$, 3 μmoles ATP, 80 μmoles of Na^+, 10 μmoles of K^+, 100 μg protein and varying concentrations of ouabain; ●–●, intact cells; ●- -●, homogenized cells.

The optimum pH for the hydrolysis of Mg^{2+}-ADP and ATP was at 7.8 (Fig. 7) in both homogenized and intact cells. There was a slight shift to a pH of 8.0 for the maximal production of P_i from Mg^{2+}-ATP in the homogenized cells incubated with Na^+ or with $Na^+ + K^+$. Neither the pH profile curves nor the specific activities of the enzyme reflected consistent change based upon the conditions of the cell culture (*e.g.* log phase of growth *vs.* stationary phase).

The apparent presence of an ADPase associated with the surface of intact cells could be explained by the generation of ATP from two molecules of ADP by a plasma membrane-associated ATP:AMP phosphotransferase (EC 2.7.4.3) and the subsequent hydrolysis of this product by the Mg^{2+}-ATPase. This possibility was tested by incubating intact fibroblasts with Mg^{2+}-ADP or ATP in the presence or absence of an ATP trapping system containing hexokinase, glucose-6-phosphate dehydrogenase, and $NADP^+$. The concentrations of these latter enzymes and $NADP^+$ were at levels adequate for the removal of the phosphate from ATP such that the product of this phosphate transferase, ADP, was now hydrolyzed at the same rate as control tubes containing the trapping system and ADP (Figs. 8A and 8B). The

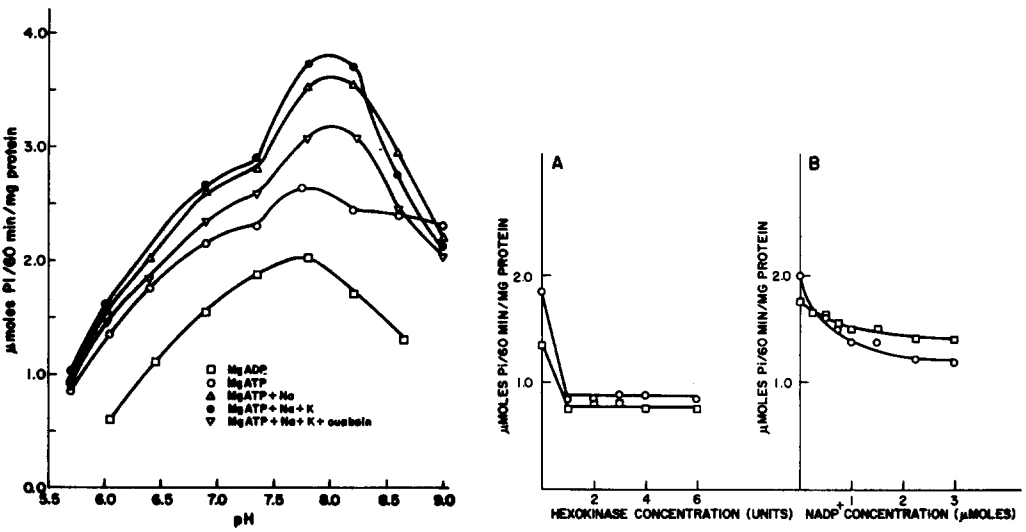


Fig. 7. The hydrolysis of ADP and ATP by homogenates of chick fibroblasts as a function of specific ions and pH. Chick embryo fibroblasts were harvested in the log phase of growth and phosphatase activities determined at pH 5.5–9.5. The cells (100 μg protein) were incubated at 37° for 60 min with 20 μmoles of "Good" buffer in the following pH ranges: pH 5.5–6.5, 2-(*N*-morpholino) ethanesulfonic acid; pH 7.0 and 7.5, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulformic acid; pH 8.0–9.5, *N,N*-bis-(2-hydroxyethyl)glycine. The specific conditions of incubations were 3 μmoles Mg^{2+} and 3 μmoles ADP (□–□); 3 μmoles Mg^{2+} and 3 μmoles ATP (○–○); 3 μmoles Mg^{2+}, 3 μmoles ATP and 80 μmoles Na^+ (△–△); 3 μmoles Mg^{2+}, 3 μmoles ATP, 80 μmoles Na^+ and 10 μmoles K^+ (●–●); and 0.5 μmole ouabain (▽–▽).

Fig. 8. The hydrolysis of ADP and ATP as a function of the concentration of hexokinase and $NADP^+$. A. Chick embryo fibroblasts were incubated for 60 min at 37° in a 1-ml volume containing 3 μmoles of $MgSO_4$, 20 μmoles Bicine buffer (pH 7.8), 180 μg protein, 20 μmoles glucose, 0.18 unit of glucose-6-phosphate dehydrogenase, 3 μmoles $NADP^+$, 3 μmoles of ADP (□–□) or ATP (○–○), sucrose to a final concentration of 250 mosM, and 0–6 units of hexokinase. B. Fibroblasts were incubated as in A, but at a constant concentration of hexokinase (1 unit) and a variable quantity of $NADP^+$ (0–3 μmoles).

nucleotide triphosphate was hydrolyzed at a greater rate than ADP in the absence of hexokinase, glucose-6-phosphate dehydrogenase, and $NADP^+$ (Table II). However, in the presence of the trapping system the ADP and ATP with its phosphate removed were hydrolyzed at the same rate (Table II). These data preclude the combination of an adenylate kinase and an ATPase as an explanation for the release of P_i from ADP, and it is likely, therefore, that the ADPase is a specific enzyme associated with the surface membrane.

TABLE II

THE HYDROLYSIS OF ADP BY INTACT FIBROBLASTS IN THE PRESENCE OR ABSENCE OF A HEXOKINASE SYSTEM

Chick embryo fibroblasts were incubated at 37° for 60 min in a 1-ml volume containing 3 μmoles $MgSO_4$, 3 μmoles ADP or ATP, 20 μmoles Bicine buffer (pH 7.8), and sucrose to a final concentration of 250 mosM. The fibroblasts which were incubated with the hexokinase system had, in addition to the mentioned components, 20 μmoles glucose, 1 unit of hexokinase, 0.18 unit of glucose-6-phosphate dehydrogenase, and 1 μmole of $NADP^+$. The mean ± S.E. was calculated from four experiments.

Substrate	*μmoles P_i released per 60 min per mg protein*	
	−Hexokinase system	*+ Hexokinase system*
ADP	1.20 ± 0.22	0.93 ± 0.20
ATP	2.36 ± 0.68	0.89 ± 0.18

The difference in the amount of P_i that was derived from ADP in the presence *versus* the absence of the trapping system may indicate that some ATP:AMP phosphotransferase was associated with the plasma membrane. However, this difference might also be accounted for by the release from lysed cells of intracellular organelles and enzymes.

The presence of a reasonably active ADPase on the cell surface of intact fibroblasts could serve as an enzyme marker for this membrane. However, before this may be assumed, it was necessary to know the distribution of this enzyme between cell membrane and the intracellular membranes and organelles. Intact cells and the same preparations broken by sonication were incubated with nucleotide tri-, di- and monophosphates and the distribution of phosphohydrolases estimated from these enzymatic activities (Tables III–V). It was assumed as a rationale for this work that the polar, nucleotide substrates do not penetrate a cell membrane and that the subsequent hydrolysis of these substrates by intact cells would be by enzymes associated only with the surface. However, cells which were lysed by enzymatic treatment or were broken by mechanical means would hydrolyze these nucleotides by enzymes on the plasma membrane and by those which are associated with intracellular membranes.

The ADPase of cultured chick fibroblasts was localized predominantly on the cell surface. There was no significant increase in the specific activity of these cell preparations as a consequence of breakage (Table III). Cytidine diphosphatase was also associated with the surface membrane, whereas the remainder of the nucleotide diphosphates (GDP, IDP and UDP) was hydrolyzed by enzymes on the cell

TABLE III

THE NUCLEOTIDE DIPHOSPHATASE ACTIVITY OF CHICK EMBRYO FIBROBLASTS

The mean ± S.E. was calculated from 3–7 experiments.

Substrate	*μmoles P_i released per 60 min per mg protein*				
	ADP	*CDP*	*GDP*	*IDP*	*UDP*
Intact cells*	2.38 ± 0.26	1.71 ± 0.26	1.15 ± 0.19	2.24 ± 0.24	2.05 ± 0.43
Broken cells**	2.46 ± 0.39	1.84 ± 0.47	3.36 ± 0.55	3.77 ± 0.97	4.19 ± 0.82
Intact/broken × 100	96.6	93.2	34.1	59.4	48.9

* Chick embryo fibroblasts were incubated at 37° for 60 min in a 1-ml volume containing 20 μmoles Bicine buffer (pH 7.8), 3 μmoles $MgSO_4$, 3 μmoles of substrate, and sucrose to a final concentration of 250 mosM.

** Cells were broken by sonication.

surface and by those associated with intracellular membranes such as the endoplasmic reticulum, mitochondria, *etc.*

The Mg^{2+}-ATPase, GTPase, and ITPase were distributed between the plasma membrane and the intracellular membrane systems (Table IV). The CTPase and UTPase activities reflected a distribution like that of ADPase and CDPase. There was some loss of enzymatic activity following sonication (*e.g.* CTP- and UTPases in Table IV, and ADPase in Table V), but this was not a consistent observation.

The monophosphatase activity of intact cells was about one-tenth that of the activity of the ADPase (Table V). Broken preparations of cells had much higher monophosphatase activity; the majority of these enzymes were presumably associated with intracellular membranes or the internal face of the plasma membrane.

TABLE IV

THE NUCLEOTIDE TRIPHOSPHATASE ACTIVITY OF CHICK EMBRYO FIBROBLASTS

Intact and broken cells were incubated and treated as in Table III. The mean ± S.E. was calculated from three experiments.

Substrate	*μmoles P_i released per 60 min per mg protein*				
	ATP	*CTP*	*GTP*	*ITP*	*UTP*
Intact cells	2.88 ± 0.11	2.89 ± 0.38	4.72 ± 0.26	3.14 ± 0.37	3.30 ± 0.53
Broken cells	4.88 ± 0.37	2.68 ± 0.31	6.57 ± 0.37	4.02 ± 0.39	3.23 ± 0.35
Intact/broken × 100	59	108	72	78	102

DISCUSSION

Specific nucleotide phosphohydrolases like 5′-AMPase (EC 3.1.3.5) and the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3) have been employed in isolation procedures[5,6,9,11] as markers for the plasma membrane. These designations were based upon electron microscopic and light microscopic observations of cytochemically

TABLE V

THE NUCLEOTIDE MONOPHOSPHATASE ACTIVITY OF CHICK EMBRYO FIBROBLASTS

Intact and broken cells were incubated and treated as in Table III. The mean ± S.E. was calculated from four experiments.

Substrate	*μmoles P_i released per 60 min per mg protein*					
	ADP	*AMP*	*CMP*	*GMP*	*IMP*	*UMP*
Intact cells	2.59 ± 0.35	0.27 ± 0.01	0.24 ± 0.08	0.29 ± 0.08	0.22 ± 0.04	0.29 ± 0.05
Broken cells	2.17 ± 0.34	0.84 ± 0.25	0.88 ± 0.27	0.64 ± 0.20	0.60 ± 0.20	0.79 ± 0.21
Intact/broken × 100	119	32	27	46	36	36

stained material and on the separation and biochemical characterization of the different membranes and organelles within cells[18]. However, because of species and tissue differences, only the ion transport ATPase is generally recognized as a marker for this membrane.

The presence of a nucleotide diphosphatase which hydrolyzes ADP in intact fibroblasts and its predominant localization on the cell membrane provide a marker enzyme of greater specificity than that of the Mg^{2+}-ATPase. This latter enzyme is associated with the intracellular membranes and organelles as well as the plasma membrane.

Morphological studies with histochemical techniques established that enzymes on the surface of epithelial cells[18,19], endothelial cells[20] and certain blood cells[21] could hydrolyze ADP. In biochemical studies, ERNSTER AND JONES[22] observed that in isolated rat liver microsomes there was a differential effect of deoxycholic acid on the enzymatic activity and the degree of solubilization of nucleotide diphosphatases which hydrolyzed ADP and CDP from others which hydrolyzed UDP, GDP, and IDP. Studies by WALLACH AND ULLREY[23] on plasma membrane isolated from Ehrlich ascites cells had also indicated that the ADPase was concentrated in a membrane fraction which was enriched in sialic acid and phospholipid. WATTIAUX-DE CONINCK AND WATTIAUX[24] isolated the plasma membrane from rat liver homogenates by flotation equilibrium centrifugation on sucrose gradients and achieved a 25-fold concentration of ADPase and 5'-AMPase. The nucleotide monophosphatase is generally considered to be a component of liver plasma membranes. The isolated membrane preparation hydrolyzed all the nucleotide diphosphates and was stimulated by both Mg^{2+} and Ca^{2+}.

Enzymes associated with the surface of the intact fibroblasts also hydrolyze all the nucleotide di- and triphosphates but to varying degrees. The capacity of the plasma membrane to hydrolyze a broad range of substrates may reflect the low specificity of the nucleotide phosphohydrolases or indicate the presence of many specific hydrolases. This latter possibility seems less likely, but in either case, the nucleotide di- and triphosphohydrolases associated with the intracellular membrane do not hydrolyze ADP, CDP, CTP and UTP. These differences in phosphohydrolase specificity of the cell membrane and the intracellular membranes and membrane systems provided markers for plasma membrane isolation studies. By use of this

criterion as well as the distribution of sialic acid, lipids, and other marker enzymes, it has been possible to isolate the plasma membrane from cultured chick embryo fibroblasts[11], from similar cells converted by oncogenic RNA viruses (J. F. PERDUE, unpublished results) and from cultured rat liver parenchymal cells (J. F. PERDUE, unpublished results).

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