HUMAN PLACENTAL ATP–DIPHOSPHOHYDROLASE: BIOCHEMICAL CHARACTERIZATION, REGULATION AND FUNCTION

A. M. KETTLUN,[1] A. ALVAREZ,[1] R. QUINTAR,[1] M. A. VALENZUELA,[1]* L. COLLADOS,[1] E. ARANDA,[2] A. BANDA,[1] L. CHAYET,[1] M. CHIONG,[1] M. MANCILLA[1] and A. TRAVERSO-CORI[1]

[1]Departmento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago [*Fax* 562-2227900] and [2]Departmento de Hematología, Facultad de Medicina, Pontificia Universidad Católica, Santiago, Chile

Abstract—1. Kinetic and physico-chemical studies on human placental microsomal fraction confirmed that the ATPase and ADPase activities detected in this fraction correspond to the enzyme ATP–diphosphohydrolase or apyrase (EC 3.6.1.5). These include substrate specificity, and coincident M_r and pI values of both ATPase–ADPase activities.

2. This enzyme hydrolyses both the free unprotonated and cation–nucleotide complex, the catalytic efficiency for the latter being considerably higher.

3. Microsomal apyrase is insensitive to ouabain and Ap5A. The highly purified enzyme was only inhibited by *o*-vanadate, DES and slightly by DCCD.

4. Apyrase seems to be a glycoprotein from its interaction with Concanavalin-A.

5. Preliminary studies on the essential amino acid residues suggest the participation of Arg, Lys and His residues, and discard the requirement of $-SH$, COO^-, $-OH$, and probably also Tyr and Trp.

6. Two kinetic modulatory proteins of apyrase were detected in placental tissue. An activating protein was found in the soluble fraction and an inhibitory protein was loosely bound to the membranes.

7. The proposed *in vivo* function for apyrase is related to the inhibition of platelet aggregation due to its ADPase activity, which is supported by the direct effect on washed platelets and by its plasma membrane localization.

INTRODUCTION

ATPase activity in human term placenta was initially demonstrated by Cerletti *et al.* (1960). But its biochemical characterization was undertaken by Miller and Berndt (1973). These authors found at least two proteins with ATPase activity, one of them was Mg^{2+}-dependent and stimulated by Na^+ and K^+, the other was a Ca^{2+}-ATPase. This latter enzyme hydrolysed ATP as well as GTP, ITP and ADP and was insensitive to ouabain. Besides Ca^{2+}-ATPase, a high affinity calcium-stimulated Mg^{2+}-ATPase has been described, whose function might be the transport and regulation of intracellular calcium concentrations (Treinen and Kulkarni, 1986; Tuan and Kushner, 1987).

During placental development both microsomal ATPases (Na^+, K^+), stimulated Mg^{2+}-ATPase and Ca^{2+}–Mg^{2+}-ATPase increase steadily with gestational age until weeks 18–21, and decrease in the second half of pregnancy. No change in K_m was observed during this period (Mukherjea *et al.*, 1986). Two components of calcium-stimulated ATP hydrolysis in human placental basal plasma membrane were also characterized by Kelley *et al.* (1990). One of them probably corresponds to the same enzyme initially studied by Miller and Berndt (1973) because of its substrate unspecificity and divalent cation stimulation, and the other could be the calcium-transporting ATPase.

*To whom correspondence should be addressed.

Abbreviations: Ap5A, adenylyl (3′–5′)-adenosine pentaphosphate; DCCD, dicyclohexylcarbodiimide; DES, diethylstylbestrol; PMFS, phenylmethylsulfonyl fluoride; DTNB, *bis*-dithionitrobenzoic acid; AMP-PCP, 5′-(β, Γ-methylene) triphosphate; TNM, tetranitromethane; WSC, water soluble carbodiimide, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide; Koshland reagent, 2-hydroxy-5-nitrobenzyl bromide.

A powerful ADPase activity that inhibited ADP-induced platelet aggregation was demonstrated in human placental extracts by Hutton *et al.* (1980). The enzyme could be a protective mechanism since significant placental thrombosis is a relatively rare phenomenom. It must be pointed out that placental tissue itself produces very small quantities of PGI_2, another antiaggregatory agent, when compared to other vascular tissues (Barradas *et al.*, 1983). Hence this ADPase activity might be replacing the PGI_2 effect. It has been established also that ADPase activity in umbilical cord plasma is significantly higher than that in the corresponding human maternal blood (Barradas *et al.*, 1990). These results indicate that ADPase activity must be produced in the placental tissue. Recently, Yagi *et al.* (1992) showed that this activity is not separated from the ATPase activity but is expressed by one enzyme, ATP–diphosphohydrolase.

Papamarcaki and Tsolas (1990) presented evidence which indicated the existence of ATP–diphosphohydrolase, known as apyrase (EC 3.6.1.5), in the placental tissue. Our research group has also been working in normal human term placental apyrase and has found similar characteristics with regard to those described by Papamarcaki and Tsolas (1990). These similarities are in relation to its hydrolytic activity on ATP and ADP, the stimulating effect of Ca^{2+} or Mg^{2+}, the inhibitory effect of azide and fluoride, the insensitivity towards ouabain and Ap5A and also in relation to the microsomal subcellular localization. Nevertheless, we have found significant differences in the phosphorylated nucleosides specificity and DCCD effect. The reason for this disagreement is not clear because in both cases the characterization was done in a microsomal preparation.

The physiological role of ATP–diphosphohydrolase in this vascular tissue is probably the same as that proposed by Miura *et al.* (1987) for the enzyme present in aortic epithelium. This function can be related to foetal blood flux regulation due to its ADPase activity, which inhibits ADP-induced platelet aggregation.

Our research group has demonstrated the presence of an apyrase-activating protein in several rat tissues (Valenzuela *et al.*, 1989). The existence of this modulatory protein indicates that *in vivo* this enzyme should be regulated.

The present work has three main aspects: a biochemical characterization of this enzyme, which includes kinetic and physico-chemical studies; a functional aspect through studies of its effect on platelet aggregation and subcellular localization, in order to support the proposed physiological role related to foetal blood flux regulation; and a regulatory aspect looking for the presence of ATP–diphosphohydrolase modulatory proteins in placental tissue.

MATERIALS AND METHODS

Materials

All the chemicals used were of analytical grade and obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) or from Merck (Darmstadt, Germany).

Microsomal fraction preparation

Microsomal fraction was prepared according to Pieber *et al.* (1991) from normal human term placenta, previously washed in isotonic saline (0.15 M NaCl) to remove the circulating blood.

This preparation was used for the biochemical and physico-chemical characterization, membrane separation and the effect on platelet aggregation. Samples taken from the foetal or maternal side of the placenta did not show any significant difference in either activity or ATPase–ADPase subcellular distribution.

Enzyme assays

Apyrase activity was measured with ATP (ATPase activity) or ADP (ADPase activity) as substrate at a final concentration of 2 mM in the presence of 0.5 or 5 mM $CaCl_2$ in 100 mM Tris–HCl, pH 8.0; the P_i (inorganic phosphate) released was determined using the method of Fiske and Subbarow (1925) or Ernster *et al.* (1950), according to the sensitivity required. Because the ampholines interfere with the Fiske and Subbarow method, the Ernster method was used for detecting apyrase activity of the enzyme eluted from isoelectrofusing gels. A unit of activity (U) is defined as the amount of enzyme which produces 1 μmol of P_i per min at 30 C.

The 5′-nucleotidase (5′-AMPase) and glucose-6-phosphatase (G-6-Pase) were measured as described by Beaufay *et al.* (1974). Glutamate and lactate dehydrogenases were determined according to Leighton *et al.* (1968) and Bergmeyer *et al.* (1974), respectively.

Protein determination

Protein was determinated by the method of Lowry *et al.* (1951), with BSA as standard.

Metal ion quantification

Calcium, magnesium and manganese contamination in the assay mixture due to the buffer, substrate and enzyme was detected by atomic absorption spectrophotometry.

Kinetic parameters determination

Apyrase K_m and V_m values for the free nucleotide were determined in the presence of 50 μM EDTA to remove the Ca^{2+} and Mg^{2+} contamination. The kinetic parameters for the calcium–nucleotide complex in the presence of 0.5 mM Ca^{2+} (maximal activity concentration) and 5.0 mM Ca^{2+} (inhibitory concentration) were also determined.

The competition plot

The competition plot described by Chevillard *et al.* (1993) consisted briefly of choosing a concentration a_0 of substrate A that gives v_0 in the absence of B, and a concentration b_0 of B that gives the same v_0 in the absence of A, were selected. Then a series of mixtures containing A and B were prepared at concentrations $a = (1-p)\ a_0$ and $b = pb_0$, respectively, and the total rate was determined for each mixture and plotted against p. The p values ranged from 1 to 0. A constant rate independent of p indicates that the two reactions occur at the same site. Substrates A and B were ATP and ADP, respectively. The identical v_0 values were obtained using 0.4 mM ATP and 0.2 mM ADP.

Partial fractionation of the microsomal membranes

One ml (13.4 mg of protein) of the microsomal fraction resuspended in the homogenization buffer (0.25 M sucrose, 5 mM $MgCl_2$ 25 mM KCl, 50 mM Tris–HCl, pH 7.5) was placed on the top of a partially continuous sucrose gradient made in 50 mM Tris–HCl, pH 7.5, and centrifuged in a SW 30 rotor at 40,000*g* for 2 hr. The sucrose gradient consisted of 5 ml of 50% sucrose, 20 ml of a linear gradient between 45 and 30% sucrose and finally 5 ml of 25% sucrose. One ml aliquots were collected and assayed for ATPase–ADPase activities, 5′-AMPase and G-6-Pase.

M_r determination

The M_r associated with the microsomal ATPase–ADPase activities was determined by the irradiation–inactivation method using ^{60}Co (Kempner and Schlegel, 1979). The freeze-dried microsomal samples in vacuum-ampoules were irradiated at the Comisión Chilena de Energía Nuclear in a ^{60}Co irradiator Naratom 3500, with an average dose rate of 417.3 Gy/h. This dose was equivalent to 1 Mrad/day. The control samples were kept at the same temperature conditions as the irradiated ones. The logarithm of the residual activity was plotted as a function of the dose; the slope of this function was determined by linear regression analysis. The inverse of this value corresponded to the 37% dose (D_{37}), and this data was used to empirically calculate the M_r. Apyrase true ATPase activity was measured in the presence of oligomycin in order to discount possible mitochondrial ATPase contamination. ADPase activity also corresponded to apyrase because Ap5A did not change the activity found.

Microsomal enzyme solubilization

n-Octylglucoside (10 mg/1 mg of protein) was added to the microsomal fractions (resuspended in 50 mM Tris–HCl, pH 7.5) and incubated for 60 min at 30°C. Solubilized proteins were separated by centrifugation at 100,000*g* for 1 hr and the supernatant was collected.

pI of the ATPase–ADPase activities

The pI was determined by isoelectrofocusing in the presence of 2 M urea according to Sánchez *et al.* (1980). Because of the high aggregability of the enzyme, 1% of *n*-octylglucoside was also added. After measuring the pH gradient, the gel was cut into 2 mm slices and the proteins eluted with 50 mM Tris–HCl, pH 8.0, containing 0.1 M NaCl and 0.5% *n*-octylglucoside. After a minimum of 3 hr or usually 24 hr, aliquots were assayed for ATPase and ADPase activities with 0.5 mM $CaCl_2$ in the assay medium.

SDS–PAGE

Electrophoresis, under denaturing conditions, was done in slabs 0.03 × 6 × 10 cm, with gel mixture as described by Laemmli (1970). The enzyme eluted from the isoelectrofocusing gel with a corresponding pI of 4.5 was concentrated 10 times using the Amicon apparatus through a Sartorius PM 10 membrane, exhaustively

dialysed against distilled water and finally freeze-dried before applying to the SDS gel.

Interaction of apyrase with lectins

SDS–PAGE was performed as described above and proteins were transferred to nitrocellulose paper according to Towbin *et al.* (1979). The enzyme transferred to the nitrocellulose filter was treated separately with four different lectins conjugated to peroxidase obtained from Sigma Chemical Co. For the Concanavalin-A–peroxidase assay the paper was blocked for an hour with 0.1 mM $CaCl_2$, 0.1 mM $MnSO_4$, 0.15 M NaCl, 3% (p/v) gelatin in 10 mM sodium phosphate, pH 6.8. For the other three lectins tested (*Erithrina cristagalli*, *Maclura pomifera* and *Tetragonolobus purpureas*), the blocking solution was 0.15 M NaCl, 3% (p/v) gelatin in 10 mM sodium phosphate, pH 6.8. Then, each nitrocellulose paper was incubated for 24 hr with the corresponding lectin diluted 1/1000 in the same buffer used for blocking but with 1% (p/v) gelatin. After incubation the paper was washed 3 times with the same buffer without gelatin. Finally the interaction was developed through the peroxidase assay using a solution containing 60 mg of 4-chloro-1-naphtol, 20 ml methanol, 60 μl 30% H_2O_2 prepared in the respective buffer without gelatin.

Platelet aggregation

Platelet aggregation was monitored following the transmittance increase as a function of time in an aggregometer PAP-4 (Biodata Corp.) according to Mustard *et al.* (1972). The medium contained citrate-treated platelet-rich human plasma (2×10^8 platelets/ml), and a final concentration of 4 μM ADP to induce aggregation. The apyrase (0.0146 U/ml) tested was eluted from an isoelectrofocusing gel with 0.1 M NaCl in 50 mM Tris–HCl, pH 8.0, and further concentrated 10 times by the Amicon apparatus through a Sartorius PM 10 membrane. In the control experiment apyrase was replaced by 0.15 M NaCl.

Chemical modification of amino acid residues

Modifications were performed as described by Means and Feeney (1964). The modification time, pH and reagent concentration employed depended on the modifier reagent. Aliquots of the treated sample were then assayed for ATPase–ADPase activities. Control experiments were performed without the specific reagent but with the corresponding modifier solvent. The buffer conditions were the following: 0.1 M Tris–HCl, pH 8.0, for DTNB and TNM modifications; 0.1 M sodium bicarbonate, pH 8.0, for phenylglyoxal reaction; 0.08 M sodium acetate, pH 4.0, for Koshland and diethylpyrocarbonate modifications; 0.09 M methyl glycine ester, pH 4.7, for WSC pre-incubation; and 0.05 M tricine, pH 9.0, for maleic anhydride reaction. Prevention of the modification was done with 10 mM AMP-PcP as substrate analogue (a competitive inhibitor of the ATPase and ADPase activities).

Partial isolation and assay of apyrase modulatory proteins

Both the 100,000*g* supernatant and the solubilized microsomal fractions (previously incubated with *n*-octylglucoside 10 mg/1 mg of protein or with 2 M NaCl) were separately precipitated with 50% ammonium sulphate. This precipitate was centrifuged at 17,300*g* for 30 min. The pellet was dissolved in 50 mM Tris–HCl, pH 7.5, to $\frac{1}{4}$ of the initial volume, heated at 100°C for 5 min (which separates inactivated apyrase and other proteins) and rapidly cooled in ice. The insoluble material produced by the heating was separated by centrifugation at 17,300*g* for 30 min. The supernatant was freeze-dried and dissolved in distilled water in $\frac{1}{10}$ of the initial volume, and then exhaustively dialysed against 0.1 M NaCl. These fractions were called Fr-50.

The presence of a regulatory protein was assayed as described in Mancilla *et al.* (1987). Briefly, an homogenous apyrase preparation purified from *S. tuberosum* tuber or a microsomal fraction obtained from placental tissue was pre-incubated with the modulatory fraction or with BSA (at the same protein concentration) as control, in the presence of 0.1 M NaCl. Aliquots of these mixtures were assayed for apyrase activity as previously described.

RESULTS AND DISCUSSION

Apyrase subcellular distribution

The ATPase–ADPase specific activities of the 100,000*g* pellet, which should correspond to apyrase, increased 7.7 times compared to the homogenate (Table 1). The recovery of these activities in this membrane fraction was close to 30%. Practically no enzymic activity was found in the final supernatant. This microsomal fraction was also enriched with 5′-AMPase (plasma

Table 1. Partial isolation of plasma membrane and endoplasmic reticulum. For experimental details see Materials and Methods section. The sucrose gradient and the respective analytical determination of activities and protein were run in duplicate

Enzymatic activity	Specific activity (U/mg protein)	Purification factor
Microsomal fraction:		
ATPase	0.176	7.7*
ADPase	0.180	7.9
5′-AMPase	0.100	5.7
G-6-Pase	0.0011	5.5
Sucrose gradient (39.5%)		
ATPase	0.690	23.1
ADPase	0.697	30.8
5′-AMPase	0.252	15.4
G-6-Pase	0.002	9.9
Sucrose gradient (24.8%)		
ATPase	0.40	17.7
ADPase	0.41	18.2
5′-AMPase	0.18	10.3
G-6-Pase	0.0051	25.3

*The purification of the enzymatic activities was calculated in relation to the homogenate.

membrane marker) and in G-6-Pase (endoplasmic reticulum marker); their increases in specific activities compared to the homogenate are also shown in Table 1. This fraction was neither contaminated with glutamate dehydrogenase (mitochrondrial marker) nor lactate dehydrogenase (cytosolic marker).

Because apyrase shows a typical microsomal distribution pattern, further separation of the different types of membranes was necessary to assure its subcellular localization. The microsomal types of membrane were practically separated by a partially continuous sucrose gradient, purity and recovery of the isolated membranes were determined by the use of 5′-AMPase and G-6-Pase. The total recovery of apyrase and G-6-Pase was approx. 95% and 5′-AMPase was 70%. A zone with ATPase–ADPase activities was found in the 39.5% sucrose layer with a larger purification of apyrase and 5′-AMPase (Table 1). G-6-Pase activity was concentrated at the 24.8% sucrose layer (Table 1). From these results we conclude that apyrase is localized in the plasma membrane and its association with endoplasmic reticulum is ruled out.

Kinetic characterization of the microsomal preparation

The competition plot. This kinetic method determines whether competitive reactions (ATPase and ADPase) occur at the same active site, or correspond to multiple sites (Chevillard *et al.*, 1993). A horizontal straight line resulted in the competition plot (i.e. a constant rate independent of p), indicating that both hydrolytic activities correspond to the same active site of one single enzyme (Fig. 1).

Apyrase ion metal dependence. The microsomal ATPase–ADPase activities are stimulated by bivalent metal ion, Ca^{2+} being the most efficient, followed by $Mg^{2+} > Mn^{2+} > Zn^{2+} > Co^{2+}$ (data not shown). Considering the

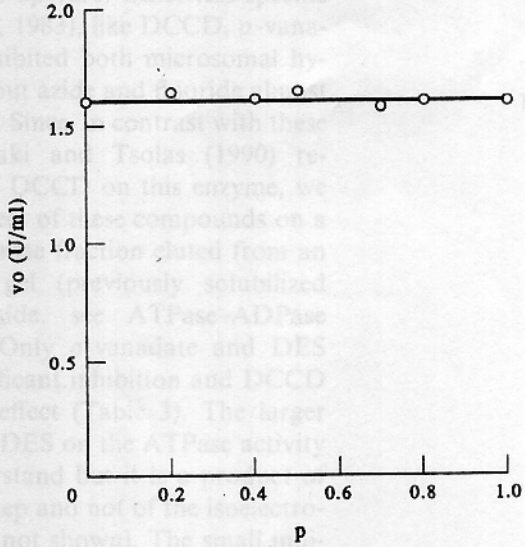

Fig. 1. Competition plot. Substrate A (ATP) at $p = 0$ was 0.4 mM; substrate B (ADP) at $p = 1$ was 0.2 mM. Assay medium also contained 5 mM $CaCl_2$ in 0.1 M Tris–HCl, pH 8.0. All the experiments were run at least in duplicate.

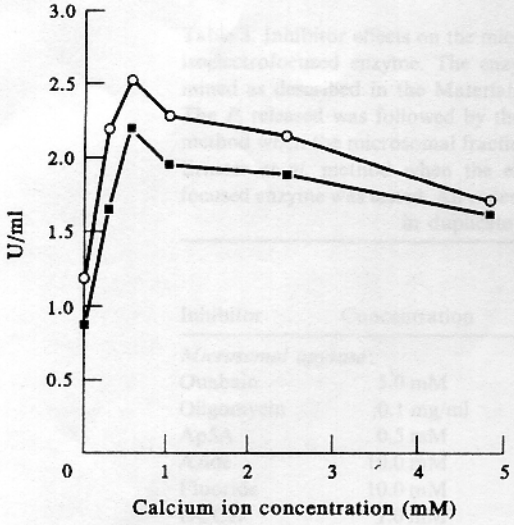

Fig. 2. Calcium ion effect on ATPase–ADPase activities. ■, ATPase; ○, ADPase. The enzyme assay was done in 50 μM EDTA and 0.1 M Tris–HCl, pH 8.0. The experiment was run at least in duplicate.

activity obtained with Ca^{2+} as 100% a relative value of *c.* 40% was reached with Co^{2+}. Apyrase activity (measured with 2 mM nucleotide) reached its maximum value at 0.5 mM Ca^{2+} (Fig. 2). In the absence of added metal ion the amount of contamination was negligible, being 10 μM for Ca^{2+} and 4 μM for Mg^{2+}, no manganese ions were detected. The addition of 50 μM EDTA, to chelate the contaminating metal ions, partially decreased the activity measured in the presence of calcium (Table 2). A further activity reduction was produced by 10 mM EDTA, EGTA or CDTA which cannot be explained by metal ion chelation, but could be due to the direct inhibitory effect of these agents on the enzyme. This non-absolute metal ion dependence makes the placental apyrase different from the enzymes of other origins (Kettlun *et al.*, 1982; Valenzuela *et al.*, 1989; Pieber *et al.*, 1991). The activity maximum reached at a lower calcium concentration than the one necessary to completely chelate the nucleotide, suggests that the free metal ion also binds to the enzyme but in a non-catalytic form. From these results we conclude that both the free nucleotide and the metal complexes are substrates. Laliberté *et al.* (1982) have already reported that ATP^{4-}, $Ca\text{-}ATP^{2-}$ and $Mg\text{-}ATP^{2-}$ were substrates of the pancreas ATP–diphosphohydrolase, but free cations did not bind to the enzyme.

Table 2. Kinetic parameters of free and complexed nucleotides. For experimental details see Materials and Methods section. All experiments were run at least in duplicate

		V_m	K_m	V_m/K_m
ATP	50 μM EDTA	0.210	0.086	2.44
ADP	50 μM EDTA	0.208	0.064	3.25
Ca–ATP	0.5 mM Ca^{2+}	0.325	0.041	7.90
Ca–ADP	0.5 mM Ca^{2+}	0.395	0.040	9.90
Ca–ATP	5.0 mM Ca^{2+}	0.254	0.088	2.90
Ca–ADP	5.0 mM Ca^{2+}	0.384	0.105	3.70

The kinetic parameters of these two species were determined. The Hanes–Woolf plots were linear, giving no indication of co-operativity or substrate inhibition in the experimental conditions used, either with 0.5 or 5.0 mM Ca^{2+}, or in the absence of contaminating metal ions (Table 2). The catalytic efficiency determined in the presence of 0.5 mM $CaCl_2$ was higher than that of the free nucleotides. The decreased value observed in the presence of an inhibitory calcium concentration (5 mM) was due to the simultaneous decrease in V_m and increase in K_m.

Effect of inhibitors. (Na^+, K^+)-ATPase in this microsomal fraction was not detected because of its insensitivity towards ouabain (Table 3). Although no mitochondrial marker activity was observed in this membrane fraction, the small inhibitory effect of oligomycin on the ATPase activity can be interpreted as a contamination by mitochondrial ATPase (Table 3). The adenylate kinase inhibitor, Ap5A (Feldhaus *et al.*, 1975), did not change ATPase–ADPase activities. These results led us to conclude that we were measuring a true ADPase activity which must correspond to apyrase. Other less specific inhibitors (Serrano, 1983), like DCCD, *o*-vanadate and DES, inhibited both microsomal hydrolytic activities, but azide and fluoride almost did not affect them. Since, in contrast with these results, Papamarcaki and Tsolas (1990) reported no effect of DCCD on this enzyme, we also studied the effect of these compounds on a highly purified apyrase fraction eluted from an isoelectrofocusing gel (previously solubilized with *n*-octylglucoside, see ATPase–ADPase isoelectric point). Only *o*-vanadate and DES caused a very significant inhibition and DCCD had only a slight effect (Table 3). The larger inhibitory effect of DES on the ATPase activity is difficult to understand but it is a product of the solubilization step and not of the isoelectrofocusing one (data not shown). The small inhibition by oligomycin, azide and fluoride, and larger effect of DCCD on the microsomal preparation was no longer observed in the solubilized enzyme. The differences compared to the

Table 3. Inhibitor effects on the microsomal and solubilized isoelectrofocused enzyme. The enzyme activity was determined as described in the Materials and Methods section. The P_i released was followed by the Fiske and SubbaRow method when the microsomal fraction was used, and by the Ernster *et al.* method when the effect on the isoelectrofocused enzyme was tested. All experiments were run at least in duplicate

Inhibitor	Concentration	Inhibition (%) ATPase	ADPase
Microsomal apyrase:			
Ouabain	5.0 mM	0	0
Oligomycin	0.1 mg/ml	18.6	0
Ap5A	0.5 mM	0	0
Azide	10.0 mM	12.4	28.1
Fluoride	10.0 mM	18.0	26.7
DCCD	1.0 mM	33.0	30.0
o-Vanadate	0.1 mM	30.3	36.3
DES	1.0 mM	63.0	57.2
Isoelectrofocused apyrase:			
DCCD	1.0 mM	12.4	11.2
o-Vanadate	0.1 mM	70.0	61.3
DES	1.0 mM	72.9	50.4

membrane-bound apyrase could be due to some conformational changes produced on the solubilized enzyme due to the absence of the membrane hydrophobic portion.

Amino acid residues participation in the enzymatic reaction. The participation of COO^-, -OH and -SH groups is excluded because of apyrase insensitivity towards WSC, PMSF and DTNB, respectively. The effects of the different amino acid modifier reagents are summarized in Table 4.

The enzyme activity decayed when Tyr and Trp residues were modified with TNM and Koshland reagent, respectively, indicating that both groups were modified. The lack of protection by AMP-PcP (substrate analogue) suggests that these residues are not involved at the active site. The inactivation enhancement observed when the Koshland reagent and AMP-PcP were added together, can be interpreted as a conformational change produced by the AMP-PcP enzyme binding which exposed some Trp residues. The loss of ATPase–ADPase activities of the human placental preparation, when either of the aromatic residues were modified, was probably produced by structural changes of the enzyme caused by chemical modification of these two amino acids, which indirectly affected the active site.

The data shown in Table 4 support the participation of Arg, Lys and His residues. The inactivation observed by the addition of phenylglyoxal (Arg modifier) was largely avoided when the enzyme was pre-incubated with AMP-PcP. The large decrease in activity produced by maleic anhydride, which is relatively selective for amino groups, together with the partial protection by AMP-PcP, could also suggest that Lys residues might be involved at the active site. It is known that Arg generally serves as an anion binding site, particularly for phosphate and carboxylate groups (Riordan, 1973); also Lys residues have been considered to be involved in nucleotide binding and catalysis (Hollenberg *et al.*, 1971). The non-absolute dependence of this apyrase for bivalent metal ions could rest in the known pyrophosphate neutralizing role of Arg, thus facilitating nucleophilic attack on the free nucleotide (Cotton *et al.*, 1973).

The loss of activity produced by diethylpyrocarbonate (His modifier) was prevented by AMP-PcP. The participation of His and Lys

Table 4. Chemical modification of ATP-diphosphohydrolase. Controls were preincubated with the respective solvent used for solubilization of the reagents. AMP–PcP (substrate analogue) had a final concentration of 10 mM. Buffer conditions are described in the Materials and Methods section. All experiments were run at least in duplicate

Reagent	Concentration (mM)	Preincubation time (min)	Temperature (°C)	Inactivation (%) ATPase	ADPase
TNM	100	30	30	67	70
+AMP-PcP	100	30	30	62	64
Koshland R.	20	10	20	61	65
+AMP-PcP	20	10	20	85	85
DEP	100	15	30	70	67
+AMP-PcP	100	15	30	21	24
Phenylglyoxal	20	60	20	65	77
+AMP-PcP	20	60	20	16	30
Maleic anhydrate	16	15	0	76	73
+AMP-PcP	16	15	0	58	54

residues was supported by the pH profile of both ATPase–ADPase activities, where pK_a values around 7.5 and 9.5 were interpolated (data not shown).

These chemical modification studies on the human placental apyrase show some substantial differences from the other enzymes already studied. In homogenous *S. tuberosum* and rat placental apyrases, Tyr and Trp groups are probably involved in the substrate binding site, and carboxylic groups in the catalytic site (Kettlun *et al.*, 1982; Pieber *et al.*, 1991). A deprotonated His residue could function as a general basic catalyser or a nucleophile instead of Asp or Glu.

In spite of the differences just mentioned, there are some similarities too, such as the unessentiality of the −SH groups and the possible participation of Arg and His (Kettlun *et al.*, 1982, 1992). The requirement of amino groups seems doubtful in plant apyrase (Valenzuela *et al.*, 1973), and perhaps also in the placental enzyme because of the low substrate analogue protection from maleylation (Table 4).

Physico-chemical characterization

Enzyme solubilization. To determine the apyrase M_r and pI, the enzyme was released from the membrane with *n*-octylglucoside. Both ATPase–ADPase activities recovery was close to 80%. Almost no activity was detected in the pellet remaining after 100,000*g* centrifugation. This solubilized protein is very stable frozen or at 4°C. Other detergents tested were Triton X-100 or Tween 20 but they inactivated the enzyme and Brij 58 did not solubilize apyrase from the membrane. Both the high solubilization and stability in the glycosidic detergent indicate that perhaps we are dealing with a glycoprotein.

ATPase–ADPase molecular mass. Placental-solubilized apyrase showed a large tendency to aggregate, which complicated the M_r determination by gel filtration. The ATPase–ADPase activities eluted in the void volume even though disaggregating agents (urea, Brij 58 or *n*-octylglucoside) were added. This large aggregability probably means that it is a highly hydrophobic protein. A similar situation was found with the enzyme isolated from rat mammary gland (Valenzuela *et al.*, 1989), but it is not the general case.

To overcome this difficulty the radiation–inactivation technique was employed. This technique reveals the size of the smallest unit required to carry out the enzyme function (Kempner and Schlegel, 1979). Classical target analysis data have been used to determine the M_r of enzymes in the native membranes. The inactivation of ATPase–ADPase activities occurred as a simple exponential function of radiation dose. Molecular sizes of 62,700 for ATPase and 60,500 for ADPase were determined. The similar M_r values obtained for ATPase and ADPase activities support the hypothesis that both belong to the same enzyme protein. The high stability of this hydrolytic enzyme is remarkable; the loss of activity of the radiation–inactivation controls kept 10 days at room temperature was only 7%.

The M_rs found are in accordance with the ones reported for other mammalian apyrases determined by gel filtration, such as the enzymes from pig pancreas (LeBel *et al.*, 1980), from some rat tissue (Valenzuela *et al.*, 1989; Pieber *et al.*, 1991) and from human umbilical vessels (Yagi *et al.*, 1992), but differ from the value of 110 kDa described for the bovine aorta apyrase (Yagi *et al.*, 1989). However Côté *et al.* (1991), using the same ^{60}Co radiation–inactivation method, calculated M_rs of 132 kDa and 189 kDa for the pancreas and aorta enzymes, respectively. The data discrepancies show that there are different types of tissue-dependent diphosphohydrolases.

ATPase–ADPase isoelectric point. A protein band of pI 4.5 hydrolysed both ATP and ADP in the presence of $CaCl_2$. This eluted fraction showed a main protein band of M_r close to 64 kDa by SDS–PAGE. This value agrees with that found by the irradiation–inactivation technique. This fraction was sometimes contaminated with two other minor bands with each one representing no more than 10% of the total protein. One of these has a M_r of 98 kDa and the other a M_r of 210 kDa. The activity recovery after isoelectrofocusing was approx. 30% and was stable either frozen or at 4°C. The elution of the protein with the same buffer but without detergent allowed a recovery of only 10%. The substrate specificity and the effect of some inhibitors (see "Inhibitors effect") were studied with this highly purified enzyme preparation. In addition to ATP and ADP, all the nucleoside di-and triphosphates tested (GTP, CTP, TTP, UTP, GDP, CDP, TDP and UDP) were hydrolysed at rates between 50 and 100% compared to ATP or ADP. These specificity data were similar to those obtained using the

Table 5. Placental thermostable proteins and potato calmodulin on placental microsomal and potato soluble apyrase. For experimental details see the Materials and Methods section. All experiments were run at least in duplicate

Sample tested	Concentration (mg/ml protein)	Activity (%)			
		Placental enzyme		Potato enzyme	
		ATPase	ADPase	ATPase	ADPase
BSA	the same as sample	100	100	100	100
Soluble Fr-50	1.0	124	120	150	160
Microsomal Fr-50	0.17	84	83	78	77
Potato calmodulin	1.0	100	100	100	100

microsomal fraction. The enzyme eluted from the gel did not have phosphomonoesterase activity. The pI value seems to depend on the enzyme origin because the rat placental apyrase is a very basic protein of pI 9.36 (Pieber *et al.*, 1991).

Apyrase interaction with lectins. Our group has previously reported the interaction of rat placental apyrase with Concanavalin A (Pieber *et al.*, 1991), which suggests that the enzyme is glycosylated. There is evidence that other mammalian apyrases are glycoproteins, like the enzyme isolated from pig pancreas (LeBel *et al.*, 1980) and from human umbilical vessels (Yagi *et al.*, 1992). The placental apyrase high solubility and further stability in *n*-octyglucoside pointed to the presence of carbohydrate moieties in this protein too. In order to have an insight into the type of carbohydrate linked to this protein, we tested the possible interaction of the enzyme with four lectins: Concanavalin A, *Erythrina cristagalli*, *Maclura pomifera* and *Tetragonolobus purpureas*. These glycoproteins have different sugars. This study, followed through the positive peroxidase reaction, showed that only Concanavalin A with α-D-man and α-D-glc groups interacted with the isoelectrofocused apyrase. This result indicates that the enzyme might have at least one of these two different types of sugars and additionally rules out the presence of gal, fuc and glcNAc moieties. The presence of a glycoprotein of M_r close to 65 kDa detected in the SDS–PAGE developed with dansyl hydrazine (fluorescent detection) also supports the possibility that apyrase is glycosylated.

Kinetic regulation of ATP-diphosphohydrolase

Our research group has previously found in the *S. tuberosum* tuber, the presence of an apyrase-activating and an inhibitory protein (Mancilla *et al.*, 1987). Later we detected a stimulatory protein in the cytosolic fraction of rat mammary gland and uterus (Valenzuela *et al.*, 1989). In the present work both Fr-50 prepared from the cytosolic and microsomal fractions of the placental tissue were analysed (Table 5). The cytosolic Fr-50 had an apyrase stimulatory effect in both the plant homogeneous and in placental microsomal enzymes. On the other hand, the microsomal Fr-50 prepared after NaCl protein solubilization, produced an inhibitory effect on both apyrases. The microsomal proteins solubilized with *n*-octylglucoside did not have any modulatory action on apyrase. These results could mean that the ligand forces of the inhibitory protein to the membrane are through electrostatic interactions or that it is a soluble protein too, unspecifically or weakly bound to membranes. The activating effect is not due to the possible presence of the known heat stable calmodulin, because homogeneous potato calmodulin prepared in our laboratory (Mancilla *et al.*, 1987) did not change the apyrase activity of either of the two enzymes tested (Table 5).

The existence of apyrase kinetic modulatory proteins in the diverse tissues studied, suggests the fact that this enzyme may have a clue metabolic function. We found that the enzyme levels changed during *S. tuberosum* tuber maturation (Anich *et al.*, 1990) and recently detected changes in apyrase activity in rat uterus and mammary gland during the lactogenic cycle (Valenzuela *et al.*, 1992). Uterine activity decreased during pregnancy and increased in the mammary gland in the lactation period. These findings support our hypothesis that this enzyme is a highly regulated protein, making even more interesting the study of its physiological role in the different tissues described so far, including plants and animals.

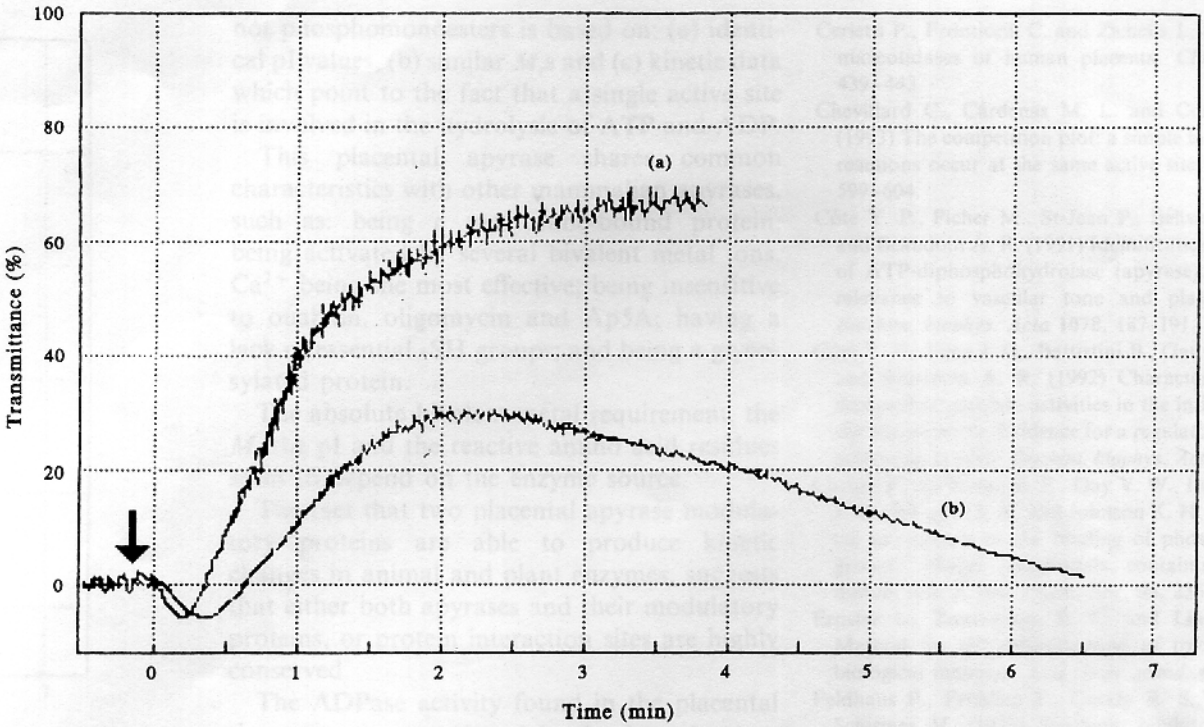

Fig. 3. Effect of placental apyrase on ADP-induce platelet aggregation. (a) Aggregation control in response to 4 μM ADP and (b) addition of 40 μl of the isoelectrofocused apyrase containing 0.0042 U of ADPase activity in a final volume of 288 μl.

Apyrase functional studies

Inhibition of ADP-induced platelet aggregation. The first research group to relate the function of this enzyme with an inhibitory effect on platelet aggregation was Ribeiro *et al.* (1984) working with blood-sucking mosquitoes. This was later confirmed by Yagi *et al.* (1991) showing that bovine aorta ATP–diphosphohydrolase is an ecto-enzyme both in endothelial and smooth muscle cells. The *in vitro* experiments of the placental microsomal fraction or the highly purified apyrase on platelet aggregation agreed with this function. The inhibitory effect of the purified sample on ADP-dependent platelet aggregation is shown in Fig. 3. The enzyme caused not only a large decrease in aggregation but also a reversal of the process, which implied that both the external ADP added and the internal ADP released by the aggregated platelets were hydrolysed. The assessment of the physiological function of removing the blood circulating ADP proposed for this enzyme should be done through the demonstration that apyrase is an ecto-enzyme. The presence of ecto-apyrase in erythrocytes (Lüthje *et al.*, 1988), synaptosomes (Schadeck *et al.*, 1989; Rocha *et al.*, 1990) and bovine aorta endothelial and smooth muscle cells (Yagi *et al.*, 1991) has already been reported. Côté *et al.* (1992) suggested a dual role for this enzyme located in the intima and media layers of bovine aorta in regulating platelet activation: by converting ATP released from damaged vessel cells into ADP, thus inducing platelet aggregation at the sites of vascular injury and then by converting ADP released from aggregated platelets and/or from haemolysed erythrocytes to AMP. The same enzyme could inhibit or reverse platelet activation, limiting the growth of platelet thrombus at the site of the injury.

CONCLUSIONS

The actual existence of apyrase or ATP–diphosphohydrolase in human placenta is confirmed by the biochemical characterization of the ATPase–ADPase activities of this tissue. The demonstration that a single protein hydrolyses nucleoside di- and triphosphates but

not phosphomonoesters is based on: (a) identical pI values, (b) similar M_rs and (c) kinetic data which point to the fact that a single active site is involved in the hydrolysis of ATP and ADP.

This placental apyrase shares common characteristics with other mammalian apyrases, such as: being a membrane-bound protein; being activated by several bivalent metal ions, Ca^{2+} being the most effective; being insensitive to ouabain, oligomycin and Ap5A; having a lack of essential -SH groups; and being a glycosylated protein.

The absolute bivalent metal requirement, the M_r the pI and the reactive amino acid residues seem to depend on the enzyme source.

The fact that two placental apyrase modulatory proteins are able to produce kinetic changes in animal and plant enzymes, suggests that either both apyrases and their modulatory proteins, or protein interaction sites are highly conserved.

The ADPase activity found in the placental tissue, as was mentioned in the Introduction, seems necessary to compensate for the diminished PGI_2 production (Barradas *et al.*, 1983). The decrease in placental ADPase described in cases of intrauterine growth retardation supports the significative role of apyrase in maintaining good foetal nutrition (O'Brien *et al.*, 1987).

Acknowledgements—This work was financed by Grants: Fondecyt No. 90-1006, DTI 3345-9213 and Universidad de Chile PG 029-92. We thank Sr Juan Espinoza from the Comisión Chilena de Energía Nuclear who kindly carried out the irradiation–inactivation experiments.

REFERENCES

Anich M., Fanta N., Mancilla M., Kettlun A. M., Valenzuela M. A. and Traverso-Cori A. (1990) Apyrase activity and changes in metabolites during germination and tuberization of *Solanum tuberosum*. *Phytochemistry* **29,** 1411–1415.

Barradas M. A., Craft I. L., Dandona P. and Jeremy J. Y. (1983) Does the human placenta produce prostacyclin? *J. Physiol.* **336,** 77.

Barradas M. A., Mikhailidis D. P. and Dandona P. (1990) ADPase activity in human maternal and cord blood: possible evidence for a placenta-specific vascular protective mechanism. *Int. J. Gynecol. Obstetr.* **31,** 15–20.

Beaufay H., Amar-Costesec A., Feytmans E., Thines-Sempoux D., Wibo M., Robbi M. and Berthat J. (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. *J. Cell Biol.* **61,** 188–200.

Bergmeyer H. V., Gawehn K. and Grassed M. (1974) *Methods in Enzymatic Analysis*, Vol. 1, pp. 425–522. Academic Press, London.

Cerletti P., Fronticelli C. and Zichella L. (1960) Adenosine nucleotidases of human placenta. *Clin. chim. Acta* **5,** 439–443.

Chevillard C., Cárdenas M. L. and Cornish-Bowden A. (1993) The competition plot: a simple test of whether two reactions occur at the same active site. *Biochem. J.* **289,** 599–604.

Côté Y. P., Picher M., St-Jean P., Béliveau R., Potier M. and Beaudoin A. R. (1991) Identification and localization of ATP-diphosphohydrolase (apyrase) in bovine aorta: relevance to vascular tone and platelet aggregation. *Biochim. biophys. Acta* **1078,** 187–191.

Côté Y. P., Filep J. G., Battistini B., Gauvreau J., Sirois P. and Beaudoin A. R. (1992) Characterization of ATP-diphosphohydrolase activities in the intima and media of the bovine aorta: Evidence for a regulatory role in platelet activation *in vitro*. *Biochim. biophys. Acta.* **1139,** 133–142.

Cotton F. A., Hazen E. E., Day V. W., Larsen S., Norman J. G., Wong S. T. K. and Johnson K. H. (1973) Biochemical importance of the binding of phosphate by arginyl groups. Model compounds containing methylguanidinium ion. *J. Am. Chem. Soc.* **95,** 2367–2369.

Ernster L., Zetterström R. C. and Lindberg O. (1950) Method for the determination of tracer phosphate in biological material. *Acta chem. scand.* **4,** 942–947.

Feldhaus P., Frohlich T., Goody R. S., Isahow M. and Schirmer H. (1975) Synthetic inhibitors of adenylate kinases in the assays for ATPase and phosphokinases. *Eur. J. Biochem.* **57,** 197–204.

Fiske C. H. and Subbarow Y. (1925) The colorimetric determination of phosphorus. *J. biol. Chem.* **66,** 375–400.

Hollenberg P. F., Flashner M. and Coon M. J. (1971) Role of lysyl ϵ-amino groups in adenosine diphosphate binding and catalytic activity in pyruvate kinase. *J. biol. Chem.* **246,** 946–953.

Hutton R. A., Dandona P., Chow F. P. R. and Craft I. L. (1980) Inhibition of platelet aggregation by placental extracts. *Thromb. Res.* **17,** 465–471.

Kelley L. K., Borke J. L., Verma A. K., Kumar R., Penniston J. T. and Smith C. H. (1990) The calcium-transporting ATPase and the calcium- or magnesium-dependent nucleotide phosphatase of human placental trophoblast basal plasma membrane are separate enzyme activities. *J. biol. Chem.* **265,** 5453–5459.

Kempner E. S. and Schlegel W. (1979) Size determination of enzymes by radiation inactivation. *Analyt. Biochem.* **92,** 2–10.

Kettlun A. M., Uribe L., Calvo V., Silva S., Rivera J., Mancilla M., Valenzuela M. A. and Traverso-Cori A. (1982) Properties of two apyrases from *Solanum tuberosum*. *Phytochemistry* **21,** 551–558.

Kettlun A. M., Urra R., Leyton M., Valenzuela M. A., Mancilla M. and Traverso-Cori A. (1992) Purification and characterization of two isoapyrases from *Solanum tuberosum* var. Ultimus. *Phytochemistry* **31,** 3691–3696.

Knowles A. F., Isler R. E. and Reece J. F. (1983) The common ocurrence of ATP-diphosphohydrolase in mammalian plasma membranes. *Biochim. biophys. Acta* **731,** 88–96.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriphage T4. *Nature* **227,** 680–685.

Laliberté J. F., St-Jean P. and Beaudoin A. R. (1982) Kinetic effects of Ca^{2+} and Mg^{2+} on ATP hydrolysis by the purified ATP diphosphohydrolase. *J. biol. Chem.* **257,** 3869–3874.

LeBel D., Poirier G. G., Phaneuf S., St-Jean P., Laliberté J. F. and Beaudoin A. R. (1980) Characterization and purification of a calcium-sensitive ATP diphosphohydrolase from pig pancreas. *J. biol. Chem.* **255,** 1227–1233.

Leighton F., Poda B., Beaufay H., Baudhuin P., Coffey J. W., Fowler S. and De Duve C. (1968) The large scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* **37,** 482–513.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

Lüthje J., Schomburg A. and Ogilvie A. (1988) Demonstration of a novel ecto-enzyme on human erythrocytes, capable of degrading ADP and of inhibiting ADP-induced platelet aggregation. *Eur. J. Biochem.* **175,** 285–289.

Mancilla M., Valenzuela M. A., Anich M., Kettlun A. M., Jara O. and Traverso-Cori A. (1987) Identification of an apyrase activating protein and of calmodulin in *Solanum tuberosum*. *Phytochemistry* **26,** 2471–2474.

Means G. E. and Feeney R. E. (1964) *Chemical Modification of Proteins*. Holden–Day, San Francisco, Calif.

Miller R. K. and Berndt W. O. (1973) Evidence for Mg^{2+}-dependent, $Na^+ + K^+$-activated ATPase and Ca^{2+}-ATPase in the human term placenta. *Proc. Soc. exp. biol. Med.* **143,** 118–122.

Miura Y., Hirota K., Arai Y. and Yagi K. (1987) Purification and partial characterization of adenosine diphosphatase activity in bovine aorta microsomes. *Thromb. Res.* **46,** 685–695.

Mukherjea M., Chakraborti A. S. and Misra S. (1986) Characterization of microsomal ATPases from developing human placenta. *Biochem. Med. Metabol. Biol.* **35,** 115–119.

Mustard J. F., Perry D. W., Ardlie N. G. and Packham M. A. (1972) Preparation of suspensions of washed platelet from humans. *Br. J. Haematol.* **22,** 193–204.

O'Brien W. F., Knuppel R. A., Saba H. I., Angel I. L., Benoit R. and Bruce A. (1987) Platelet inhibitory activity in placentas from normal and abnormal pregnancies. *Obstetr. Gynecol.* **70,** 597–600.

Papamarcacki T. and Tsolas O. (1990) Identification of ATP diphosphohydrolase activity in human term placenta using a novel assay for AMP. *Molec. cell. Biochem.* **97,** 1–8.

Pieber M., Valenzuela M. A., Kettlun A. M., Mancilla M., Aranda E., Collados L. and Traverso-Cori A. (1991) ATPase–ADPase activities of rat placental tissue. *Comp. Biochem. Physiol.* **100B,** 281–285.

Ribeiro J. M., Sarkis J. J. M., Rossignol P. A. and Spielman A. (1984) Salivary apyrase of *Aedes aegypti*: characterization and secretory fate. *Comp. Biochem. Physiol.* **79B,** 81–86.

Riordan J. F. (1973) Arginyl residues and anion binding sites in proteins. *Molec. cell. Biochem.* **26,** 71–92.

Rocha J. B. T., Mello C. F., Sarkis J. J. F. and Dias R. D. (1990) Undernutrition during the preweaning period changes calcium ATPase and ADPase activities of synaptosomal fractions of weanling rats. *Br. J. Nutr.* **63,** 273–283.

Sánchez G., Gajardo M. and De Ioannes A. (1980) IgM-like natural hemagglutinin from rat fish serum: isolation and physicochemical characterization (*Callorhynchus callorhynchus*). *Dev. comp. Immunol.* **4,** 667–678.

Schadeck R. J. C., Sarkis J. J. F., Dias R. D., Araujo H. M. M. and Souza D. O. G. (1989) Synaptosomal apyrase in the hypothalamus of adult rat. *Braz. J. med. biol. Res.* **22,** 303–314.

Serrano R. (1973) Purification and reconstitution of the proton pumping ATPase of fungal and plant plasma membrane. *Arch. Biochem. Biophys.* **227,** 1–8.

Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **109,** 173–184.

Treinen K. A. and Kulkarni A. P. (1986) High-affinity, calcium-stimulated ATPase in brush border membranes of the human term placenta. *Placenta* **7,** 365–373.

Tuan R. S. and Kushner T. (1987) Calcium-activated ATPase of the human placenta: identification, characterization, and functional involvement in calcium transport. *Placenta* **8,** 53–64.

Valenzuela M. A., Del Campo G., Marín M. and Traverso-Cori A. (1973) Effects of protein-modifying reagents on an isoenzyme of potato apyrase. *Biochem J.* **133,** 755–763.

Valenzuela M. A., López J., Depix M., Mancilla M., Catalán L., Chiong M., Garrido J. and Traverso-Cori A. (1989) Comparative subcellular distribution of apyrase from animal and plant sources. Characterization of microsomal apyrase. *Comp. Biochem. Physiol.* **93B,** 911–919.

Valenzuela M. A., Collados L., Kettlun A. M., Mancilla M., Lara H., Puente J., Aranda E., Chayet L., Alvarez A. and Traverso-Cori A. (1992) Changes in apyrase activity in uterus and mammary gland during lactogenic cycle. *Comp. Biochem. Physiol.* **103B,** 113–118.

Yagi K., Arai Y., Kato N., Hirota K. and Miura Y. (1989) Purification of ATP diphosphohydrolase from bovine aorta microsomes. *Eur. J. Biochem.* **180,** 509–513.

Yagi K., Shinbo M., Hashizume M., Shimba L. S., Kurimura S. and Miura Y. (1991) ATP-diphosphohydrolase is responsible for ecto-ATPase and ecto-ADPase activities in bovine aorta endothelial and smooth muscle cells. *Biochem. biophys. Res. Commun.* **180,** 1200–1206.

Yagi K., Kato N., Shinbo M., Shimba L. S. and Miura Y. (1992) Purification and characterization of adenosine diphosphatase from umbilical vessels. *Chem. Pharm. Bull.* **40,** 2143–2146.