Salivary apyrase of *Rhodnius prolixus*

Kinetics and purification

João J. F. SARKIS,* Jorge A. GUIMARÃES† and José M. C. RIBEIRO‡

*Department of Biochemistry, Federal University of Rio Grande do Sul, Instituto de Biosciencias, 90.000 Porto Alegre, RS, Brazil, †Department of Biochemistry, Federal University of Rio de Janeiro, Centro de Ciencias da Saude, Bloco H, 21.910 Rio de Janeiro, RJ, Brazil, and ‡Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, U.S.A.

The salivary apyrase activity of the blood-sucking bug *Rhodnius prolixus* was found to reside in a true apyrase (ATP diphosphohydrolase, EC 3.6.1.5) enzyme. The crude saliva was devoid of 5'-nucleotidase, inorganic pyrophosphatase, phosphatase and adenylate kinase activities. ATP hydrolysis proceeded directly to AMP and P_i without significant accumulation of ADP. K_m values for ATP and ADP hydrolysis were 229 and 291 μM respectively. K_i values for ATP and ADP inhibition of ADP and ATP hydrolysis were not different from the K_m values, and these experiments indicated competitive inhibition. Activities were purified 126-fold by combined gel filtration and ion-exchange chromatography procedures with a yield of 63%. The purified enzyme displayed specific activities of 580 and 335 μmol of P_i released/min per mg of protein for ATP and ADP hydrolysis respectively. The action of the purified enzyme on several phosphate esters indicates that *Rhodnius* apyrase is a non-specific nucleosidetriphosphate diphosphohydrolase.

INTRODUCTION

Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) was a name coined by Meyerhof (1945) to describe those enzymes found in yeast that hydrolysed ATP to AMP, releasing 2 mol of P_i /mol of the nucleotide. This type of activity was later identified in vertebrates, but the activity found in muscle resulted from the concerted action of two different enzymes, an ATPase (muscle myosin) and adenylate kinase (Kalckar, 1945). The existence of true apyrase enzymes was then challenged, although in liver Kalckar (1945) found apyrase activity without contaminating adenylate kinase. In plants, particularly in potato tubers, the activity was nevertheless well characterized and, on the basis of both kinetic and purification work, the activity is now accepted to reside in a true apyrase enzyme (Traverso-Cori *et al.*, 1965, 1970), although its function remains unknown.

More recently independent research has started to describe apyrase activity in both vertebrates (LeBel *et al.*, 1980; Laliberte & Beaudoin, 1983; Hamlyn & Senior, 1983; Knowles *et al.*, 1983) and invertebrates (Ribeiro & Garcia, 1980; Smith *et al.*, 1980; Mant & Parker, 1981; Ribeiro *et al.*, 1984b, 1985a). In vertebrates, the activity is well characterized in rat and pig pancreas, but the role of such activity has been only speculated upon. In invertebrates, the activity is associated with the salivary gland of blood-sucking arthropods, where an anti-haemostatic role has been ascribed, with reference to the central role of ADP in platelet aggregation (Vargaftig *et al.*, 1981). Indeed, saliva or salivary-gland homogenates of such unrelated arthropods as the bug *Rhodnius prolixus* (Ribeiro & Garcia, 1981a), the mosquito *Aedes*

aegypti (Ribeiro *et al.*, 1984a), the tsetse fly *Glossina austeni* (Mant & Parker, 1981), the tick *Ixodes dammini* (Ribeiro *et al.*, 1985a), and the sandfly *Lutzomyia longipalpis* (J. M. C. Ribeiro, P. A. Rossignol & A. Spielman, unpublished work) prevent platelet aggregation *in vitro* and contain very high concentrations of the enzymic activity (usually 5–60 units/mg of protein in saliva or salivary-gland homogenates as compared with 0.075 unit/mg of protein in pancreas homogenates) (Ribeiro & Garcia, 1980; LeBel *et al.*, 1980; Ribeiro *et al.*, 1984a).

Because these arthropods evolved independently to haematophagy and apyrase is not found at such high activity in other non-haematophagous animals, including the male partners of mosquitoes (Rossignol *et al.* 1984), it was proposed that such activities in blood-sucking arthropods were a case of convergent evolution. Probably an enzyme having a 'domestic' metabolic role in salivary glands of non-haematophagous arthropods evolved to the status of a secretory product upon development of the blood-feeding habit (Ribeiro *et al.*, 1984b). Some basic kinetic characteristics are shared by vertebrate apyrase and the salivary apyrase of *R. prolixus*: a similar ratio of rates of ATP to ADP hydrolysis, and a requirement for bivalent cations (Ribeiro & Garcia, 1980; Smith *et al.*, 1980; LeBel *et al.*, 1980; Knowles *et al.*, 1983). The study of arthropod apyrases may thus contribute to our understanding of the general role of this enzyme in cellular metabolism. Accordingly, we investigated whether the apyrase activity of *R. prolixus* results from a true apyrase enzyme. In particular, we purified the activity and studied its kinetic properties.

Abbreviation used: $C_{12}E_8$, octaethylene glycol mono-n-dodecyl ether.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Nucleotides and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bio-Gel P-300 was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). DEAE-Sephadex was obtained from Pharmacia (Uppsala, Sweden). The detergent $C_{12}E_8$ was obtained from Nikkol (Tokyo, Japan). All other reagents were of analytical grade. $[^{32}P]P_i$ was obtained from the Brazilian Institute of Atomic Energy (Sao Paulo, Brazil) and was purified by extraction as the phosphomolybdate complex into a mixture of benzene and 2-methylpropan-1-ol, and final precipitation as the $MgNH_4PO_4$ salt (Kanazawa & Boyer, 1973). $[\gamma\text{-}^{32}P]ATP$ was prepared by the method of Glynn & Chappell (1964), with small modifications (De Meis, 1972). The $[\gamma\text{-}^{32}P]ATP$ obtained was neutralized with Tris and stored at $-6^\circ C$. The ATP concentration was determined spectrophotometrically (Bock & Ling, 1956). $[\beta\text{-}^{32}P]ADP$ was prepared from $[\gamma\text{-}^{32}P]ATP$ in a reaction medium containing 50 mM-Tris/HCl buffer, pH 7.5, 1 mM-AMP, 1.5 mM- $[\gamma\text{-}^{32}P]ATP$ and 4 μ l of adenylate kinase (EC 2.7.4.3) (5 mg/ml). Incubation proceeded for 4 h at $30^\circ C$ and was stopped by immersing the test tube in a boiling-water bath for 30 s. Resulting nucleotides were separated by ion-exchange chromatography in a Dowex AG-1 $\times$ 10 (formate form) column (Cohn, 1957). ADP fractions were pooled and repurified by barium salt precipitation. De-ionized water was used throughout.

Harvesting of saliva

This was done as described in Ribeiro & Garcia (1980). Some 4000–5000 all-stage and adult bugs, starved for 2–4 weeks, were allowed to probe water for 3 min through a thin latex membrane in a warm feeding apparatus. The solution containing saliva was freeze-dried and stored desiccated at $-27^\circ C$. When needed, saliva was reconstituted with water or buffer to the desired protein concentration.

Determination of protein

Protein was assayed by the method of Lowry *et al.* (1951) or by the Coomassie Blue method of Spector (1981). In both methods bovine serum albumin was used as standard.

Assay of apyrase activity

Reaction media, unless otherwise stated, contained 30 mM-Tes/NaOH buffer, pH 7.5, 150 mM-NaCl, 1 mM- $CaCl_2$ and 1 mM-ATP or -ADP in a final volume of 0.3 ml. Reaction was initiated by the addition of 1–5 μ l of saliva and was carried out at $30^\circ C$. Incubation times were chosen to assure linearity of the velocity of product formation. The reaction was stopped by the addition of trichloroacetic acid to give a final concentration of 5% (w/v). P_i was determined in the supernatant by the Fiske & SubbaRow (1925) method. When a radioactive substrate $[\gamma\text{-}^{32}P]ATP$ or $[\beta\text{-}^{32}P]ADP$ was used, activity was determined after liquid-scintillation counting of the released $[^{32}P]P_i$ extracted as the phosphomolybdate complex into an organic phase. Reaction was stopped with addition of 0.02 ml of 75% trichloroacetic acid, followed by 0.2 ml of 0.6 M- H_2SO_4 containing 5% (w/v) ammonium molybdate, 0.2 ml of acetone and 1.0 ml of butyl acetate. Tubes were vigorously vortex-mixed, and

after phase separation a sample of the organic phase was used for liquid-scintillation counting of radioactivity. One unit of enzyme activity is defined as the amount of enzyme that releases 1 μ mol of P_i /min under the above specified standard conditions.

Assay of adenylate kinase activity

Adenylate kinase activity was determined in a reaction medium containing 30 mM-Tes/NaOH buffer, pH 7.5, 150 mM-NaCl, 1 mM- $CaCl_2$, 10 mM-glucose, 0.2 mM-NADP⁺, 10 units of Hexokinase (EC 2.7.1.1), 5 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 60 μ g of salivary protein, reactions being initiated by the addition of ATP (1 mM) or ADP (2 mM); the final volume was 1 ml. In some experiments P^{15}_5 -di-(adenosine-5')-pentaphosphate (10 μ M) was also present. Reduction of NADP⁺ was monitored spectrophotometrically at 340 nm.

Assay of pyrophosphatase activity

The procedure followed by the same as that used for the assay of apyrase activity, except that the nucleotide was replaced by PP_i (1 mM).

Analysis of apyrase reaction products

Reaction was performed as described for the assay of apyrase activity. At the indicated times reaction was stopped by addition of 0.015 ml of 100% trichloroacetic acid. Portions (20 μ l) were analysed for nucleotides by h.p.l.c. with a μ Bondapak C_{18} (Millipore) column (10 cm $\times$ 0.8 cm) equilibrated with 20% (v/v) acetonitrile in aq. 5 mM-tetrabutylammonium phosphate, pH 7.2, at a flow rate of 4 ml/min. Nucleotides were detected spectrophotometrically at 260 nm (Bock & Ling, 1956).

Gel filtration

Bio-Gel P-300 was packed in a 50 cm $\times$ 1 cm column and equilibrated with 25 mM-Tris/HCl buffer, pH 7.5, containing 0.3 M-NaCl and 0.02% NaN_3 at a flow rate of 3 ml/h. Freeze-dried saliva samples were resuspended into running buffer and centrifuged at 2000 *g* for 15 min before being applied to the column. Experiments were performed at room temperature, but eluates of the column were collected at $2^\circ C$. The tubes receiving the column eluate contained the neutral detergent $C_{12}E_8$, a procedure that prevented inactivation of apyrase activity.

Ion-exchange chromatography

DEAE-Sephadex A-50 (particle size 40–120 μ m) was equilibrated with 25 mM-Tris/HCl buffer, pH 7.5, containing 50 μ g of $C_{12}E_8$ /ml and 0.02% NaN_3 in a 13 cm $\times$ 0.8 cm glass column. Pooled fractions from the gel-filtration experiment were diluted 10-fold in starting ion-exchange buffer. Elution was performed at room temperature. A linear gradient of 20–200 mM-NaCl in 20 mM-Tris/maleate buffer, pH 6.5, containing 50 μ g of $C_{12}E_8$ /ml and 0.02% NaN_3 was applied at a flow rate of 15 ml/h.

RESULTS

Previous reports on *Rhodnius* apyrase (Ribeiro & Garcia, 1980; Smith *et al.*, 1980) did not exclude enzyme combinations that could result in apparent apyrase activity without the presence of a specific apyrase enzyme. The kinetics of product formation and substrate

Fig. 1. Reaction products of ATP hydrolysis by *Rhodnius prolixus* apyrase

ATP was incubated with *Rhodnius* saliva and at indicated times 20 μ l portions were removed and analysed by h.p.l.c. Reaction conditions are described in the Materials and methods section. $\square$, ATP; $\circ$, ADP; $\triangle$, AMP.

Fig. 2. Absence of adenylate kinase activity from *Rhodnius prolixus* saliva

The reaction medium contained 30 mM-Tes buffer, pH 7.5, 150 mM-NaCl, 1 mM-CaCl₂, 10 mM-glucose, 0.2 mM-NADP⁺, 10 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase and 60 μ g of *Rhodnius* saliva/ml and 2 mM-ADP ($\circ$) or 1 mM-ATP ($\bullet$), added as indicated by the arrow; the final volume was 1 ml. Reduction of NADP⁺ was monitored at 340 nm.

hydrolysis can give valuable information on possible enzyme combinations. After the addition of *Rhodnius* saliva to ATP, the AMP concentration increased linearly with time at least up to 60% substrate hydrolysis. The ADP concentration remained low and did not vary significantly during the same time interval (Fig. 1). This finding makes it unlikely that a combination of ATPase and adenylate kinase could be responsible for the observed

Fig. 3. Inhibition by ADP of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis by *Rhodnius prolixus* saliva

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was incubated with *Rhodnius* saliva and the velocity of $[\text{P}^{32}]\text{P}_i$ release was measured. A double-reciprocal plot of reaction velocity as a function of ATP concentration in the absence and in the presence of 50 μ M, 100 μ M- and 200 μ M-ADP is shown.

Fig. 4. Inhibition by ATP of $[\beta\text{-}^{32}\text{P}]\text{ADP}$ hydrolysis by *Rhodnius prolixus* saliva

$[\beta\text{-}^{32}\text{P}]\text{ADP}$ was incubated with *Rhodnius* saliva and the velocity of $[\text{P}^{32}]\text{P}_i$ release was measured. A double-reciprocal plot of reaction velocity as a function of ADP concentration in the absence and in the presence of 200 μ M-, 300 μ M- and 400 μ M-ATP is shown.

apyrase activity, because the equilibrium constant of the adenylate kinase reaction is near unity (Eggleson & Hems, 1952) and thus significant accumulation of ADP would have to occur. In addition, no adenylate kinase activity could be detected in *Rhodnius* saliva (Fig. 2), and the potent adenylate kinase inhibitor $\text{P}^{1}\text{P}^5\text{-di(adenosine-5')-pentaphosphate}$ (Lienhard & Secemski, 1973) at a concentration of 10 μ M did not affect either ATP or ADP

Table 1. K_m and K_i values for ATP and ADP hydrolysis by *Rhodnius prolixus* salivary apyrase

Values are given as arithmetic means, with ranges and numbers of determinations in parentheses

Nucleotide	K_m (μM)	K_i (μM)
ATP	229 (170–280; $n = 3$)	210 (200–215; $n = 3$)
ADP	290 (250–330; $n = 2$)	272 (257–287; $n = 2$)

Fig. 5. Kinetics of thermal inactivation of *Rhodnius prolixus* salivary apyrase

The thermal-inactivation medium contained 30 mM-Tes buffer, pH 7.5, 150 mM-NaCl, 1 mM- $CaCl_2$ and *Rhodnius* saliva at 1 mg of protein/ml. After the indicated times of incubation at 60 °C, samples were removed and stored on ice until determination of both ATPase (○) and ADPase (□) activities by colorimetric assay of P_i release.

hydrolysis by saliva (results not shown). The possibility of the combination of an ATP pyrophosphatase and an inorganic pyrophosphatase was excluded because no hydrolysis of PP_i was obtained when 1 mM- $Na_4P_2O_7$ was included in the reaction medium instead of ATP (results not shown). There remains the possibility of the concerted activity of an ATPase and an ADPase.

To investigate the possible association of an ATPase with an ADPase in *Rhodnius* saliva, steady-state kinetics for the hydrolysis of these two substrates were studied. ADP competitively inhibited hydrolysis of the γ -phosphate group from ATP (Fig. 3), and ATP, in an analogous fashion, inhibited ADP hydrolysis (Fig. 4). Apparent K_m and K_i values for each nucleotide are not significantly different (Table 1). The coincidence of K_m and K_i values makes it probable that only one enzyme is being responsible for the hydrolysis of both nucleotides, although the existence of an ATPase and an ADPase with coincident kinetic constants is still possible.

If the two different enzymes ATPase and ADPase coexist in *Rhodnius* saliva, then dissociation of such activities should be obtained when saliva is submitted to several physicochemical treatments. As shown in Fig. 5, thermal inactivation of ATPase and ADPase activities

Fig. 6. Elution profile of protein and enzymic activity of *Rhodnius prolixus* saliva gel-filtered through a Bio-Gel P-300 column

A 12.5 mg sample of salivary protein was applied to the column (50 cm $\times$ 1 cm) previously equilibrated with 25 mM-Tris buffer, pH 7.5, containing 300 mM-NaCl and 0.02% NaN_3 . Fractions (1 ml) were collected at a flow rate of 3 ml/h at 20 °C. Δ , Protein; $\circ$, ATPase activity; $\square$, ADPase activity. Abbreviations for inset: BSA, bovine serum albumin; FFA, *Escherichia coli* alkaline phosphatase.

presented the same pattern in each case. Further, when saliva was submitted to gel filtration followed by ion-exchange chromatography, parallel purification of both activities was achieved. Accordingly, gel filtration of saliva in Bio-Gel P-300 yielded a coincident peak of activity for ATP and ADP hydrolysis (Fig. 6). Retention in the column indicated an apparent M_r of 83000. Activities were purified about 20-fold (Table 2), with a total column recovery of 87%. Parallelism was again observed when the activities pooled from the Bio-Gel P-300 were further purified through DEAE-Sephadex chromatography (Fig. 7). This step further purified the activities 6.5-fold. Specific activities of 580 and 335 units/mg of protein for ATP and ADP hydrolysis respectively were obtained with the final purified material (Table 2). It should be noted that throughout purification the ATPase/ADPase ratio remained constant (Table 2). The enzyme thus purified is unstable and loses activity rapidly. These experiments demonstrate that salivary ATPase and ADPase activities display a parallel behaviour under thermal inactivation, gel filtration and ion-exchange chromatography.

A further kinetic confirmation for the presence of a single apyrase enzyme was sought with the method of mixed substrates (Dixon & Webb, 1979). Both crude and Bio-Gel + DEAE-Sephadex-purified preparations were incubated in standard reaction mixtures containing 1 mM-ATP, 1 mM-ADP or 1 mM-ATP + 1 mM-ADP. In the presence of a single enzyme rates of P_i production in the mixed-substrate reaction should be close to the average value found for each individual substrate, whereas if two enzymes existed the rate for the mixed-substrate reaction should be the sum of the values found for the individual substrates. Results of this experiment

Table 2. Purification of *Rhodnius prolixus* salivary apyrase

For experimental details see the text.

Procedure	Total activity (units/ml)		Protein (mg/ml)	Specific activity (units/mg)		ATPase/ADPase activity ratio	Yield (%)	Purification (fold)
	ATPase	ADPase		ATPase	ADPase			
Crude saliva	57.5	33.8	12.5	4.6	2.7	1.7	100	1
Bio-Gel P-300	50.4	29.2	0.55	92	53	1.73	87.6	20
DEAE-Sephadex	36.0	20.8	0.062	580	335	1.73	62.5	126

Fig. 7. Elution profile of protein and enzymic activity of the pooled fractions from the Bio-Gel P-300 column applied to a DEAE-Sephadex anion-exchange column (13 cm × 0.8 cm)

Elution was performed with a linear gradient (70 ml) of 20–200 mM-NaCl in 20 mM-Tris/maleate buffer, pH 6.5, containing 0.02% NaN₃ and 50 µg of C₁₂E₈/ml. ○, ATPase activity; □, ADPase activity; ----, protein concentration; —, salt gradient.

Table 3. *Rhodnius prolixus* salivary apyrase activities of crude and purified preparations in the presence of 1 mM-ATP, 1 mM-ADP or 1 mM-ATP + 1 mM-ADP

For experimental details see the text. For the experiment with ATP+ADP the values in parentheses represent the mean and the sum respectively of the activities with ATP and ADP individually.

Substrate	Apyrase specific activity (units/mg)	
	Crude preparation	Purified preparation
ATP	5.3	464
ADP	3.0	256
ATP+ADP	4.5 (4.2; 8.3)	384 (360; 720)

support the existence of a single apyrase enzyme (Table 3).

In order to identify the substrate specificity of *Rhodnius* apyrase, several nucleotides and phosphate

Table 4. Substrate specificity of purified *Rhodnius prolixus* salivary apyrase

For experimental details see the text.

Substrate	Relative velocity	P _i released (mol)
ATP	1.0	2
GTP	0.4	2
ITP	0.4	2
CTP	0.08	2
UTP	1.0	2
ADP	0.57	1
GDP	0.47	1
IDP	0.54	1
CDP	0.07	1
UDP	0.85	1
AtetraP	0.85	3
Cyclic AMP	0.0	—
AMP	0.0	—
<i>p</i> -Nitrophenyl phosphate	0.0	—
<i>p</i> -Nitrophenyl-AMP	0.0	—
Tripolyphosphate	0.0	—
Phosphate glass	0.0	—

esters were incubated with the purified enzyme. The results indicate that the enzyme is a non-specific nucleosidetriphosphate diphosphohydrolase (Table 4).

DISCUSSION

The results presented in this paper characterize the presence of a true nucleotide diphosphohydrolase enzyme in the saliva of the blood-sucking bug *Rhodnius prolixus*. Possible enzyme combinations that could lead to an apparent apyrase activity as an artifact were excluded. Adenylate kinase activity was excluded on the basis of the kinetics of ATP hydrolysis (Fig. 1), the absence of ATP production when saliva was incubated with ADP (Fig. 2) and the insensitivity of the hydrolysis reaction to P¹P⁵-di(adenosine-5′)-pentaphosphate. A combination of ATPase and ADPase was excluded on the basis of both kinetic studies and extensive parallelism observed for the hydrolytic activities of the nucleotides when saliva was submitted to a variety of treatments. Pyrophosphatase activity was excluded because saliva was not able to hydrolyse PP_i (Table 4).

It may be noted that heat treatment of saliva led to a

similar complex decay curve for both ATP and ADP hydrolysis (Fig. 3). Although this might suggest the presence of more than one enzyme with different heat-sensitivities, such behaviour has also been found for purified lysozyme (Perry & Wetzell, 1984) and may result from partial denaturation of the enzyme to a less active, more heat-resistant, intermediate.

The kinetics of hydrolysis of ATP (Fig. 1) indicates that, similarly to the pig pancreas apyrase (Laliberte & Beaudoin, 1983), *Rhodnius* apyrase may remove both phosphate groups from ATP with a single substrate contact. This explains why ADP does not accumulate in the reaction medium while the reaction proceeds directly from ATP to AMP and P_i . Apparent K_m values for both ATP and ADP are of the same order of magnitude, as well as their K_i values (Table 1), and thus if all ADP were released by the enzyme during initial hydrolysis of ATP the ADP should accumulate as a consequence of the higher concentration of ATP. These results support the following scheme of reaction for ATP hydrolysis:

This scheme is compatible with the mixed-substrate reaction result (Table 3), the absence of inhibition by accumulated AMP and P_i (as observed by the linearity of AMP production as a function of time when most of the substrate is hydrolysed; Fig. 1) and the pattern of competitive inhibition displayed by ADP and ATP (Figs. 3 and 4).

The mixed-substrate experiment resulted in hydrolysis rates that were 7% larger than the arithmetic mean of the rates in the presence of individual substrates (Table 3). Since the K_m values for ATP and ADP are approx. 0.25 mM (Table 1), at 1 mM substrate the enzyme would be 80% saturated, according to Michaelis-Menten kinetics. In the presence of 2 mM substrate, the predicted saturation would be 88.9%. Thus in the presence of mixed substrates the expected enzyme activity should be approx. 10% higher than the average of the individual rates. The values reported are in close agreement with the kinetic predictions for a single enzyme hydrolysing both ATP and ADP substrates.

The role of *Rhodnius* apyrase is clearly related to its anti-haemostatic activity, a function that facilitates blood finding during probing (Ribeiro & Garcia, 1981b). In fact, ADP released by injured cells is one of the main physiological stimuli for platelet aggregation (Vargaftig *et al.*, 1981), and *Rhodnius* saliva can effectively prevent platelet aggregation *in vitro* (Ribeiro & Garcia, 1981a). A similar role for salivary apyrase activity has been proposed for a tsetse fly (Mant & Parker, 1981), mosquitoes (Ribeiro *et al.*, 1984a, 1985b), a sandfly (J. M. C. Ribeiro, P. A. Rossignol & A. Spielman, unpublished work) and a tick (Ribeiro *et al.*, 1985a). It is interesting that these unrelated arthropods evolved towards the development of high activities of this enzyme in their salivary glands, and this is therefore an example of convergent evolution. It is possible that in these arthropods an apyrase enzyme existed before the habit of haematophagy developed, but with a function in cell metabolism still unknown.

Plant and vertebrate apyrases have no known function. In vertebrates, pancreas apyrase has been demonstrated to be an ecto-enzyme (Hamlyn & Senior, 1983). These vertebrate ecto-apyrases could degrade pharmacologically active extracellular nucleotides, as hypothesized by Burnstock (1972). Another possible general role of apyrase may be related to the intracellular catabolism of nucleotides. Several glycosyltransferase reactions are commonly inhibited by the resulting nucleoside diphosphate (Sadler *et al.*, 1982). Apyrase could enhance these reactions by removing inhibitory products, a role suggested before for microsomal nucleoside diphosphate phosphohydrolases (Ernster & Jones, 1962). Such a role of apyrase may not be restricted to animal cells. Both sucrose synthesis and starch synthesis in plants involve reversible reactions releasing UDP or ADP as products (Preiss, 1982), and apyrase may drive those reactions to completion. This effect would be similar to that of inorganic pyrophosphatase, which drives the formation of activated amino acids in protein synthesis and of carbamoyl phosphate in the urea cycle, among others (Lehninger, 1975). In support of this role of plant apyrase is the abundance of this enzyme in starch-rich plant parts such as potato tubers and peas (Traverso-Cori *et al.*, 1970; Tognoli & Marre, 1981). Thus ancestral roles for the arthropod salivary secretory apyrases may have been related to the catabolism of purinergic neuromediators or nucleotide end products of cellular metabolism.

If we consider the above proposed ubiquity of apyrase in nucleotide catabolism, we may then understand how unrelated organisms such as a tick, a mosquito, a sandfly, a tsetse fly and a blood-sucking bug have independently evolved towards the development of such high apyrase activity in their salivary glands. It is possible that their ancestors had an apyrase enzyme in their salivary glands that served a metabolic role, and later such activity was promoted from a contaminant in salivary secretion into a major secretory product upon evolution of the blood-sucking habit.

This work was supported in part by grants from Financiadora de Estudos e Projetos (FINEP-Brazil) and from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), by Grant DAMD 17-84-C-4003 from the U.S. Army Research and Development Command and by Grant AI 18694 from the U.S. National Institute of Allergy and Infectious Diseases. J. J. F. S. was the recipient of a CAPES/PICD (Brazil) fellowship.

REFERENCES

- Bock, R. M. & Ling, N. S. (1956) Arch. Biochem. Biophys. **62**, 253-264
- Burnstock, G. (1972) Pharmacol. Rev. **24**, 509-581
- Cohn, W. E. (1957) Methods Enzymol. **3**, 724-743
- De Meis, L. (1972) Biochemistry **11**, 2460-2465
- Dixon, M. & Webb, E. C. (1979) Enzymes, 3rd edn., pp. 72-75, Academic Press, New York
- Eggleston, L. V. & Hems, R. (1952) Biochem. J. **52**, 156-160
- Ernster, L. & Jones, L. C. (1962) J. Cell Biol. **15**, 563-578
- Fiske, C. H. & SubbaRow, Y. (1925) J. Biol. Chem. **66**, 375-400
- Glynn, I. M. & Chappell, J. B. (1964) Biochem. J. **90**, 147-149
- Hamlyn, J. M. & Senior, A. E. (1983) Biochem. J. **214**, 59-68
- Kalckar, H. M. (1945) J. Biol. Chem. **153**, 355-373
- Kanazawa, T. & Boyer, P. D. (1973) J. Biol. Chem. **248**, 3163-3172

- Knowles, A. F., Isler, R. E. & Reece, J. F. (1983) *Biochim. Biophys. Acta* **731**, 88–96
- Laliberte, J. F. & Beaudoin, A. R. (1983) *Biochim. Biophys. Acta* **257**, 3869–3874
- LeBel, D., Poirier, G. G., Phaneuf, S., St-Jean, P., Laliberte, J. F. & Beaudoin, A. R. (1980) *J. Biol. Chem.* **255**, 1227–1233
- Lehninger, A. L. (1975) *Biochemistry*, 2nd edn., pp. 410–411, Worth, New York
- Lienhard, G. E. & Secemski, I. I. (1973) *J. Biol. Chem.* **248**, 1121–1123
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mant, M. J. & Parker, K. R. (1981) *Br. J. Haematol.* **48**, 601–608
- Meyerhof, O. (1945) *J. Biol. Chem.* **157**, 105–119
- Perry, L. J. & Wetzell, R. (1984) *Science* **226**, 555–557
- Preiss, J. (1982) *Annu. Rev. Plant Physiol.* **33**, 431–454
- Ribeiro, J. M. C. & Garcia, E. S. (1980) *J. Insect Physiol.* **26**, 303–307
- Ribeiro, J. M. C. & Garcia, E. S. (1981a) *Experientia* **37**, 384–386
- Ribeiro, J. M. C. & Garcia, E. S. (1981b) *J. Exp. Biol.* **94**, 219–230
- Ribeiro, J. M. C., Rossignol, P. A. & Spielman, A. (1984a) *J. Exp. Biol.* **108**, 1–7
- Ribeiro, J. M. C., Sarkis, J. J. F., Rossignol, P. A. & Spielman, A. (1984b) *Comp. Biochem. Physiol. B* **79**, 81–86
- Ribeiro, J. M. C., Makoul, G. T., Levine, J., Robinson, D. R. & Spielman, A. (1985a) *J. Exp. Med.* **161**, 332–344
- Ribeiro, J. M. C., Rossignol, P. A. & Spielman, A. (1985b) *J. Insect Physiol.* **31**, 689–692
- Rossignol, P. A., Ribeiro, J. M. C. & Spielman, A. (1984) *Am. J. Trop. Med. Hyg.* **33**, 17–20
- Sadler, E., Beyer, T. A., Oppenheimer, C. L., Paulson, J. C., Priels, J.-P., Rearick, J. I. & Hill, R. L. (1982) *Methods Enzymol.* **83**, 458–514
- Smith, J. J. B., Cornish, R. A. & Wilkes, J. (1980) *Experientia* **36**, 898–900
- Spector, T. (1981) *Anal. Biochem.* **86**, 142–146
- Tognoli, L. & Marre, E. (1981) *Biochim. Biophys. Acta* **642**, 1–14
- Traverso-Cori, A., Chaimovich, H. & Cori, O. (1965) *Arch. Biochem. Biophys.* **137**, 133–142
- Traverso-Cori, A., Traverso, S. & Reyes, H. (1970) *Arch. Biochem. Biophys.* **109**, 173–184
- Vargaftig, B. B., Chignard, M. & Benveniste, J. (1981) *Biochem. Pharmacol.* **30**, 263–271

Received 25 June 1985/28 August 1985; accepted 11 October 1985