Erythrocyte 2,3 DPG and ATP concentrations and lactic acid production in heavy hashish smokers

Time (min)	2,3 DPG (mmole/mmole Hb)	p<	ATP (μmole/ml RBC)	p<	Lactic acid (μmole/ml RBC)	p<
0	1.17±0.06	-	1.71±0.10	-	1.54−0.09	-
15	1.10±0.04	0.0005	1.66±0.08	0.01	1.48−0.10	0.025
30	1.04±0.04	0.0005	1.54±0.07	0.0005	1.34−0.07	0.0005
45	0.93±0.02	0.0005	1.34±0.04	0.0005	1.25−0.05	0.0005
60	1.16±0.05	NS	1.62±0.06	0.0005	1.42−0.10	0.0005

Values are expressed as mean±SD.

cyte glycolysis. A possible explanation for this interesting finding is that of Schurr et al.[8], who proposed that for hashish smokers a temporary halt in glucose transport from the plasma to the erythrocytes occurs. This could explain the effective concentrations of the drugs in inhibiting glucose transport and correlates with the doses leading to physiological reactions. According to Schurr et al.[8] the decreased concentration of ATP, 2,3 DPG and lactic acid production observed in the present study could be explained by the low rate of efflux of glucose into erythrocytes and consequently a reduced amount of substrate for anaerobic glycolysis. However, the possibility that these findings are associated with the influence of hashish constituents on enzymes regulating the erythrocyte glycolytic rate cannot be excluded.

The reduced concentration of erythrocyte ATP is of great interest. It is well known that ATP is involved in the biosynthesis of phosphatidic acid intermediates in the production of phospholipids[14]. The reduced concentration of ATP supports our previous observations of a decreased phospholipid concentration in erythrocytes 30–60 min after hashish smoking[3]. Since it is clear that phospholipids play a functional and structural role in membrane integrity and are probably correlated with the changes observed after hashish smoking[3,9], it is evident that reduced ATP concentrations are incriminated in these changes.

The reduced concentration of 2,3 DPG after hashish smoking is another interesting finding in the present study. It is well established that this intermediate product of glycolysis is the main regulator of oxygen release from hemoglobin to the tissues, shifting the oxyhemoglobin dissociation curve (ODC) to the right[15]. Thus, the decreased level of 2,3 DPG after hashish smoking probably shifts the ODC to the left resulting in tissue hypoxia. This could explain the observation that after hashish smoking the peripheral blood flow is increased[16], since it is well documented that disturbances of tissue oxygen tension lead to adaptive changes in peripheral blood flow[17].

The results of this study clearly indicate a disturbance in the erythrocyte glycolytic pathway among heavy hashish smokers and warrants further investigation to identify the possible effect of hashish constituents on enzymes regulating the rate of erythrocyte glycolysis.

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Properties of a calcium-dependent apyrase in the saliva of the blood-feeding bug, *Rhodnius prolixus*

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Summary. The saliva of the blood-feeding bug *Rhodnius prolixus* contains at least 11 proteins, and has Ca^{2+}-dependent apyrase activity. The activity has a broad pH optimum between pH 7 and 9, and is inhibited by Mg^{2+}. Polyacrylamide gel electrophoresis and gel filtration suggest the possibility of at least 2 enzymes responsible for the activity.

Rhodnius prolixus is a triatomine hemipteran which feeds exclusively on blood. In the laboratory, it will gorge on a variety of inorganic solutions if they contain μmolar concentrations of ATP[2]. It is probable that in vivo this response to ATP enables the insect to detect an adequate supply of red blood cells[3]. Recently, the saliva of *R. prolixus* has been reported to have a potent ATPase activity[4]; one effect of this is to alter the apparent sensitivity of the bug to ATP in artificial diets. Here we report some properties of the enzyme or enzymes responsible.

Material and methods. Saliva was obtained from a laboratory stock of *R. prolixus.* Heads were pulled free of the body in 5th instar larvae, and the large, pink salivary glands were removed from the heads by pulling their ducts free of

anterior attachments. Puncturing the glands under mineral oil enabled their contents to be gently squeezed out with a glass needle. Approximately 12 glands, 2 per insect, produced about 1 µl of 'concentrated' saliva. To check for possible contamination by cell debris, comparisons were made on saliva collected by allowing insects to probe through a thin rubber membrane into a buffer solution.

For chromatography, mixtures of saliva and nucleotides were plated on cellulose MN300 TLC plates, run for 90 min in a solvent of n-butanol, acetone, acetic acid, 5% ammonia, and water (35/25/15/15/10), and examined under UV-light.

Enzyme activity was expressed as inorganic phosphate released after incubation of saliva with 1 mM ADP in 50 mM Tris-HCl buffer (pH 8) containing 2.5 mM $CaCl_2$ for 1 min at 30 °C. Each assay used 20 µl of 0.1% saliva in a final volume of 250 µl. The reaction was stopped by the addition of 250 µl of 20% TCA, and inorganic phosphate was determined by a method designed to minimize errors due to further hydrolysis of ADP[5]. Protein was measured using Coomassie Blue G250[6] with bovine serum albumin as a standard and the results were checked using a modified Lowry technique[7]. ADP production from ATP was measured spectrophotometrically using a linked pyruvate kinase method[8]. However, accurate results were impossible due to inhibition of the salivary apyrase by the levels of Mg^{2+} required for pyruvate kinase activity.

10 µl of dilute saliva containing about 20 µg of protein was electrophoresed on gradient polyacrylamide gels (Pharmacia PAA 4/30) using a single buffer system of 25 mM Tris and 186 mM glycine, pH 8.6 for 2000 Vh, typically at 500 V for 4 h. The buffer was maintained at 6± 1 °C. Gels were then incubated in 50 mM HEPES-KOH buffer at pH 7.5, with 2.5 mM Ca^{2+} and either 2.5 mM ATP or ADP. Apyrase activity was then detected by treatment with 3 mM lead nitrate and 5% ammonium sulphide. Protein bands were detected by staining with 0.025% Coomassie Blue R250 in 7% aqueous acetic acid. Saliva was also filtered through a 2.6×35 cm column of Sephacryl S-200 Superfine, equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. The column was eluted with the same buffer at 30 ml · h^{-1}.

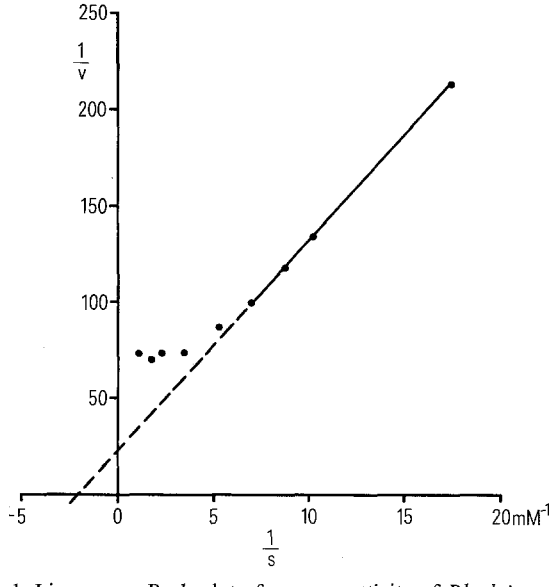

Fig. 1. Lineweaver-Burk plot of apyrase activity of *Rhodnius prolixus* saliva. Each point is the mean of 3 determinations of reaction velocity measured as P_i released after 1 min incubation of ADP with 1:12,500 saliva in Tris-HCl buffer containing 2.5 mM $CaCl_2$.

Results and discussion. Less than 1 µM ATP remained in a mixture of 1 mM ATP and 0.1% saliva in Ringer after incubation at 30 °C for 2 h. After insects had fed on 1 mM ATP in Ringer 30 min previously, sampled crop contents also showed less than 1 µM ATP. After incubation with saliva, separation of nucleotides by TLC showed both ATP and ADP to be converted to AMP; adenosine tetraphosphate, deoxyribose-ATP and uridine triphosphate were all converted to their respective monophosphates. Measurement of ADP production from ATP showed that the saliva can remove single phosphate groups from ATP as well as ADP. Salivary activity was abolished by brief boiling. These results suggest the presence in saliva of an enzyme or enzymes capable of catalyzing the removal of all except the α-phosphates from various nucleotides. Since no pyrophosphate could be found, the activity of the saliva is that of an apyrase.

Figure 1 shows a Lineweaver-Burk plot of salivary apyrase activity, using ADP as a substrate to ensure the measurement of a single reaction. The nonlinearity at high substrate concentrations is typical of reactions showing substrate inhibition. Extrapolation of the linear portion gives an apparent maximum velocity of 45 µmoles · min^{-1} mg^{-1} of protein, and an apparent K_m of 500 µM ADP. Actual maximum activities in various assays were between 12 and 15 µmoles · min^{-1} mg^{-1} protein.

The apyrase activity is calcium-dependent. There was no detectable activity in the absence of Ca^{2+}. Activity reached a maximum when Ca^{2+} and ATP (or ADP) were both at a concentration of 1 mM, and did not change significantly for up to 10 times this concentration of Ca^{2+}. Ca^{2+} cannot be substituted by Mg^{2+}, K^+ or Na^+. Mg^{2+} was somewhat

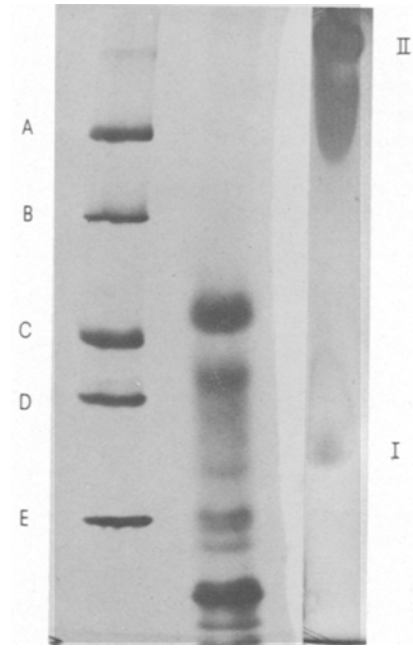

Fig. 2. Electrophoresis of saliva of *Rhodnius prolixus* on gradient polyacrylamide gels. The gels illustrated are all taken from the same slab. Saliva was loaded at the top. The slab was then sliced. Left and centre lanes were stained with Coomassie Blue to reveal proteins; and the right strip was incubated with Ca^{2+} and ADP, then stained for apyrase activity with lead nitrate and ammonium sulphide. Left: Molecular weight standards. *A* Thyroglobulin (669,000), *B* ferritin (440,000), *C* catalase (232,000), *D* lactate dehydrogenase (140,000), *E* albumin (67,000). Centre: Saliva of *R. prolixus* showing proteins. Right: Saliva, showing apyrase activity in a low (I) and high (II) molecular weight region.

inhibitory; 50 mM Mg^{2+} reduced activity in the presence of 2.5 mM Ca^{2+} to approximately 33%. 100 mM Na^+ and K^+ had no appreciable effect on activity, either alone or in combination.

There is little apyrase activity at pH 5; activity increases up to about pH 7, and remains constant between pH 7 and 9. Higher pH's were not tested.

The saliva contains approximately 250 mg protein per ml. At least 11 protein bands were observed following polyacrylamide gel electrophoresis of extracted saliva (figure 2). Apyrase activity was found in 2 positions on the gel. From a graph of the relative mobility of the protein standards vs log molecular weight, band I has a mol. wt of about 120,000 and band II has one of about 1.2×10^6 daltons. Band I activity occurred in a region containing protein bands but none were present in the region of band II. The pattern of staining and the position of the apyrase activity in the 2 gel regions was found consistently, both with saliva extracted from glands and with the buffer into which insects had salivated. The activity of both regions was calcium dependent, and was not found when heated saliva was used.

An attempt was made to separate the salivary proteins by gel filtration on Sephacryl S-200. There were 4 major peaks of absorption at 280 nm. Apyrase activity was found in 2 well separated peaks. The 1st peak, when electrophoresed, gave a band of apyrase activity in the same region as band I (figure 2). There was insufficient activity in the second peak to examine it further. No peak corresponding to band II (figure 2) was seen. A large peak, separating the two with apyrase activity, contained ferroprotoporphyrin IX, the prosthetic group of haemoglobin. This substance is associated with a pigment in the saliva derived from the haemoglobin of the blood-meal, which is probably the predominant protein of the saliva[9].

This work has clearly demonstrated the existence of at least 2 enzymes in *R. prolixus* saliva with apyrase activity, which are calcium-dependent and heat-labile. The maintained activity over high pH's might be due to 2 enzymes with overlapping pH optima. The salivary apyrase is highly active; for comparison, partly purified potato apyrase has been shown[10] to have an activity at 1 mM ATP of about 60 $\mu moles \cdot min^{-1}\ mg^{-1}$ compared to about 12 $\mu moles \cdot min^{-1}\ mg^{-1}$ for the salivary apyrase which is totally unpurified.

The function of the salivary apyrase(s) can only be speculated upon. *R. prolixus* appears to utilize ATP as a signal chemical during feeding; it should be important to remove such a signal after it had been received to avoid saturation of receptors. Alternatively, ATP might be released from damaged cells during the initial probing of the insect, and consequently may need to be removed to prevent masking of signals from the ATP in blood cells. Perhaps more likely is a role in the prevention of clotting of ingested blood. The blood meal in *R. prolixus* remains fluid for many days after feeding; the saliva has previously been shown to have anticoagulant activity[11]. Platelet aggregation is a component of the early stages of clot formation, and depends on the levels of free ATP and ADP released from activated platelets. The salivary apyrase system could maintain free ATP levels sufficiently low to prevent platelet aggregation. Perhaps relevant is the observation that tsetse flies also appear to possess a salivary apyrase[12] whereas the mosquito *Culiseta inornata* does not; blood remains fluid in tsetse flies after ingestion, but clots in mosquitoes[13]. Such a function might also be important in preventing platelet plugs forming round the mouthparts during feeding.

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Phosphorylation of human red cell and liver pyruvate kinase. Differences between liver and erythrocyte L-type subunits[1]

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Summary. Purified PK from human erythrocyte was phosphorylated by cAMP-dependent protein kinase type I from human erythrocyte membrane; this phosphorylation affected only the 'heavy' L′ subunit but not the L subunit. On the other hand, the L subunit of liver PK was highly phosphorylated. Thus it appears that the L subunits from erythrocyte and liver PK are not identical protein molecules.

Red blood cell pyruvate kinase (PK) shares several kinetic and immunologic characteristics with the liver enzyme[2-4] and it has been suggested that liver and erythrocyte enzymes might be generated from a 'common precursor subunit' (L′ type) which would be transformed into the liver type subunit (L type) by partial proteolysis[5,6]. Both 'precursor' and liver type subunits are present in erythrocytes (L'_E and L_E) with a ratio which differs from one preparation to another according to erythrocyte age and the purification method used. Phosphorylation of red cell pyruvate kinase is still open to discussion. Dahlquist-Edberg found that red cell PK in rats could not be phosphorylated by the catalytic subunit of rat liver protein kinase[7]. On the other hand Marie et al.[8] observed endogenous phosphorylation of the two subunit types of human red cell pyruvate kinase after incubation of the red cell with inorganic phosphate ^{32}P.

In this paper we demonstrate differences between the