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PROPERTIES AND SIDEDNESS OF 5'-NUCLEOTIDASE IN RAT RED CELL GHOSTS

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The occurrence of a Mg^{++}-activated 5'-AMPase activity in rat ghosts is demonstrated. This activity is inhibited by the α,β-methyleneadenosine 5'-diphosphate, a specific inhibitor of 5'-nucleotidase. The enzyme has an apparent K_m of ∿90μM, and is located on the exterior side of the plasma membrane.

5'-nucleotidase(E.C. 3.1.3.5) is a plasma membrane ectoenzyme broadly distributed in animal cells. Its function is still unclear but a functional correlation with the adenylate cyclase(1) and with the nucleoside transporting function of the membrane(2,3) are demonstrated. The enzyme is a transmembrane protein(4), and its direct interaction, age-regulated(5,6), with other proteins of the membrane is reported(7,8). Among the circulating cells its presence has been certainly demonstrated in granulocytes(9) and lymphocytes(1,10,11).

5'-nucleotidase activity has also been found in bovine(12) and human erythrocytes(13-15) although there is no agreement about that(16).

In this report the occurrence and sidedness of 5'-nucleotidase in rat erythrocyte ghosts are demonstrated.

Materials and methods

Blood from adult Wistar rats was obtained by heart puncture, and washed three times with an isotonic medium(phosphate buffer 0.155 M,pH 7.4 or NaCl 0.166 M); following this procedure the ratio leukocytes/red cells was $50/10^6$.

The white ghosts were made according to Dodge et al., as reported by Schwoch and Passow(17). The resealed ghosts were made according Schwoch and Passow(17). The preparations were stored at 4°C and all the enzyme determinations were made not later than 48h.

The concentration of ghosts was estimated from red cell count on washed red cell suspension(hematocrit 50%); the counting of the erythrocytes and leukocytes was made by visual methods.

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The acetylcholinesterase(E.C. 3.1.1.7), NADH-cytochrome *c* oxidoreductase (E.C. 1.6.99.3) and glyceraldehyde 3-phosphate dehydrogenase(E.C. 1.2.1.12) activities were assayed according Steck and Kant(18).

The 5'-nucleotidase activity was determined at 37°C using [^{32}P]AMP as substrate. In order to extrapolate the initial velocity, samples of 0.1 ml were drawn and added to 0.1 ml of 16% trichloracetic acid at fixed intervals (1 min); the ^{32}P liberated, extracted by the Sanui method(19), was determined using INSTA-GEL as scintillation mixture in the ratio extract/INSTA-GEL of 1/5(v/v), in a Packard Tricarb scintillator.

All chemicals were analytical grade. Acetylthiocholine chloride, β-NADH, β-NAD, 5,5'-dithiobis-(2-nitrobenzoic acid), cytochrome *c*, DL-glyceraldehyde 3-phosphate, 5'-AMP, α,β-methyleneadenosine 5'-diphosphate, concanavalin A, EDTA, saponin and TRITON X100 were from Sigma Chemical Co.(St.Louis,Mo.,USA). [^{32}P]AMP was from the Radiochemical Centre(Amhersham-England). INSTA-GEL was from Packard Instrument Co.(Downers Grove,Ill.,USA).

Results and discussion

The occurrence of 5'-nucleotidase activity in mammalian erythrocyte membrane is controversial.Delaunay et al.(16) did not find any activity in human red cell blood membranes. The same authors reported the absence of the above activity in erythrocytes from sheep, dog, guinea-pig, and rat. On the contrary, Parker(13), Van den Hoeck and Zail(14) and Matsumoto et al.(15) presented evidence of 5'-nucleotidase activity in human red cell ghosts, while George and Duncan(12) found it in bovine erythrocytes. An aspecific nucleotidase activity was reported in rabbit erythrocytes(20,21).

These conflicting results may be attributed to the different sensitivity of the methods used. By using a radiometric method, which allows to detect nucleotidase activities as little as pmol/h/10^6ghosts, we have shown that red cell ghosts from rat exhibit a 5'-AMPase activity. We have determined the properties and the sidedness of this enzyme in the white ghosts.

The 5'-AMP hydrolysis is linear with time up to 5 min, and with ghost concentration up to 2×10^6/µl of incubation medium(figs 1,2).

Mg^{++} ions are found to stimulate this hydrolysis, giving a maximum effect at 1.7mM(fig. 3); on the contrary the Zn^{++} ions inhibit at all concentrations tested(fig. 6). 0.1mM EDTA strongly inhibits the activity(80%-mean of three experiments).

From the pH curve(fig. 4), the optimal pH is 7.4.

The K_m determined from the kinetic curves(Hanes plots,fig. 5) is 88±10µM (mean of three experiments).

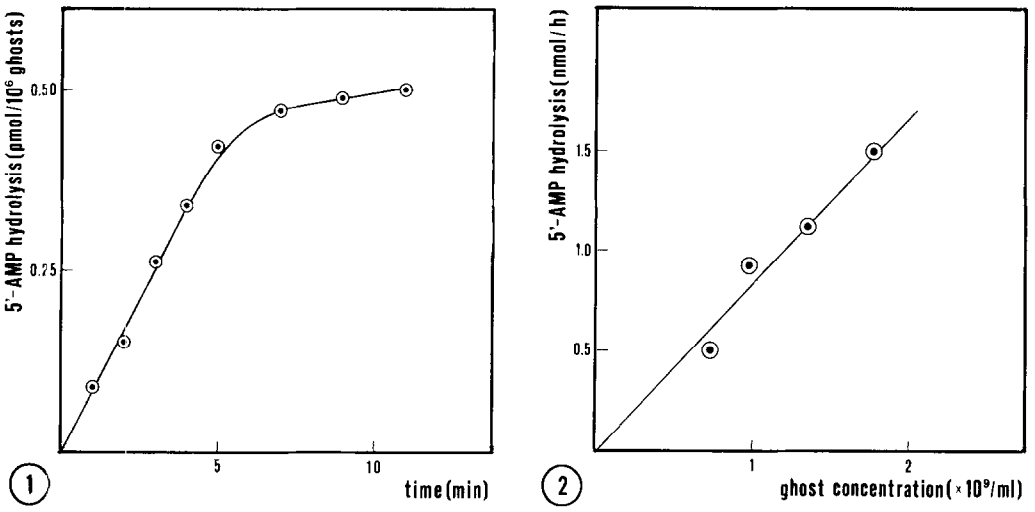


Fig. 1- Time course of 5'-AMP hydrolysis from rat white ghosts. The reaction mixture(1.5 ml) contained: TRIS-HCl 30μmol,pH 7.4, $MgCl_2$ 2.6μmol, 5'-AMP 450nmol, [^{32}P]AMP 4.5μCi, and 2.3×10^{9}ghosts. Each point represents average of 2 experiments.

Fig. 2- 5'-AMP hydrolysis as a function of rat white ghost concentration. The reaction mixture(0.6 ml) contained: TRIS-HCl 12μmol,pH 7.4, $MgCl_2$ 1.0μmol, 5'-AMP 180nmol, [^{32}P]AMP 1.8μCi, and ghosts. Each point represents average of 2 experiments.

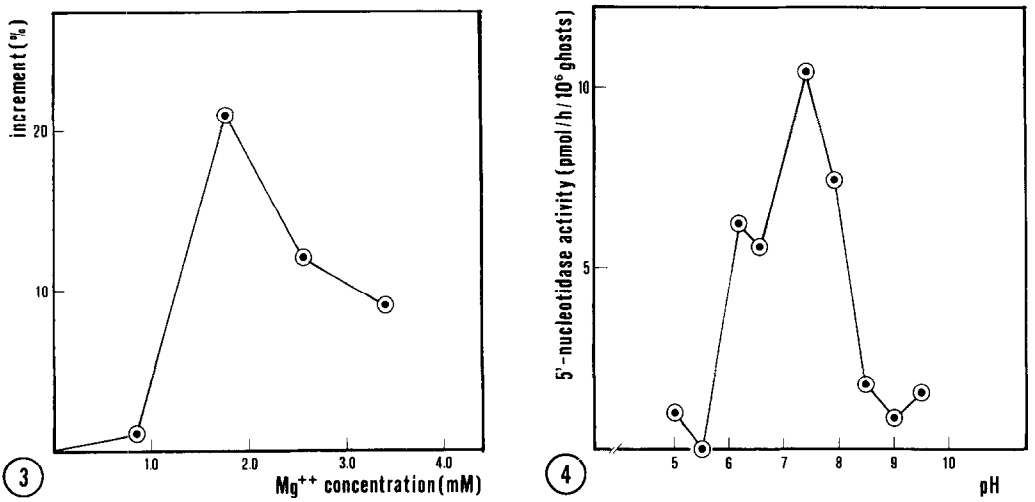
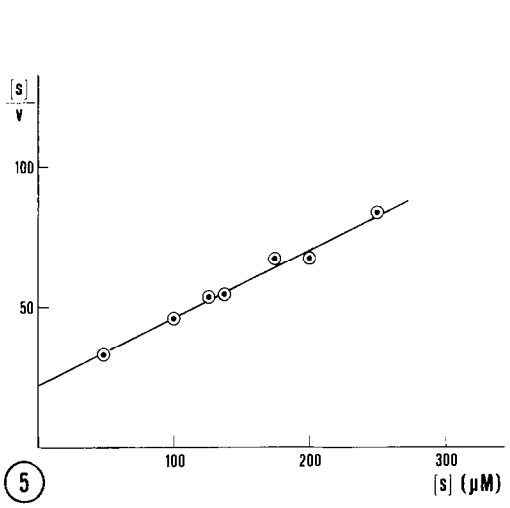


Fig. 3- Mg^{++} ions effect on 5'-nucleotidase activity of rat white ghosts. The reaction mixture(0.6 ml) contained: TRIS-HCl 12μmol,pH 7.4, 5'-AMP 180nmol, [^{32}P]AMP 1.8μCi, 1.1×10^{9}ghosts, and $MgCl_2$. Each point represents average of 2 experiments.

Fig. 4- pH curve of 5'-nucleotidase activity of rat white ghosts. The reaction mixture(0.6 ml) contained: 5'-AMP 180nmol, [^{32}P]AMP 1.8μCi, $MgCl_2$ 1.0 μmol, 0.6×10^{9}ghosts; the buffers used were: citrate 20mM,pH 5.0÷6.1, imidazole-HCl 40mM,pH 6.1÷7.4, TRIS-HCl 20mM,pH 7.4÷9.0, and carbonate-bicarbonate 20mM,pH 9.0÷9.5. Each point represents average of 2 experiments.



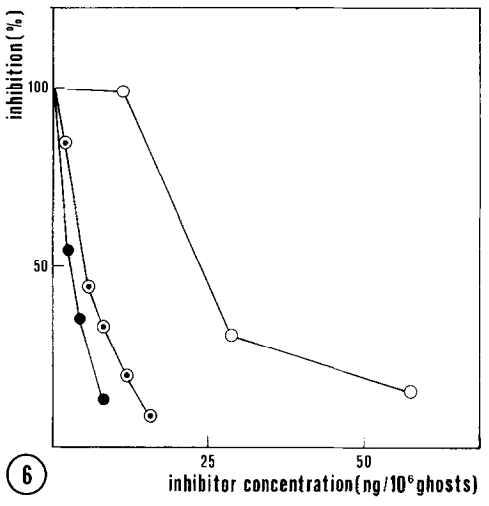


Fig. 5- Hanes plot of 5'-nucleotidase activity in rat white ghosts. The reaction mixture(0.6 ml) contained: TRIS-HCl 12μmol,pH 7.4, $MgCl_2$ 1.0μmol, [^{32}P]AMP 1.8μCi, 0.6×10^{9}ghosts and 5'-AMP 50÷250μM.

Fig. 6- Inhibition of 5'-nucleotidase activity in rat white ghosts. The reaction mixture(0.6 ml) contained: TRIS-HCl 12μmol,pH 7.4, $MgCl_2$ 1.0μmol, 5'-AMP 180nmol, [^{32}P]AMP 1.8μCi, 0.6×10^{9}ghosts and Zn^{++}(●) or α,β-methyleneadenosine 5'-diphosphate(◉) at indicated concentrations. For concanavalin A inhibition(○), the white ghosts were preincubated for 15 min at 37°C, with concanavalin A at indicated concentrations. Each point represents average of 2 experiments.

Both concanavalin A, a lectin that binds membrane glycoproteins(22), and inhibits 5'-nucleotidase from various sources(8,11,23), and α,β-methyleneadenosine 5'-diphosphate, a specific inhibitor of 5'-nucleotidase(24),inhibit the 5'-AMPase activity from white ghosts(fig. 6).

The sidedness of the enzyme has been made comparing the 5'-AMPase activity from white and resealed ghosts, either detergent-treated or not. In all preparations the accessibility of substrate on both sides of erythrocyte membrane, has been determined by using enzymatic markers: as inside markers we have used NADH-cytochrome *c* oxidoreductase(25) and glyceraldehyde 3-phosphate dehydrogenase(26), and as outside marker acetylcholinesterase(27). Both intact white and resealed ghosts exhibit the same 5'-nucleotidase activity detectable in detergent-treated preparations(fig. 7).

This finding demonstrates that 5'-nucleotidase activity of rat erythrocytes is located on the outer face of the membrane. Accordingly, Dornand et al.(28) and De Pierre and Karnovsky(9) found that 5'-nucleotidase is an ecto-

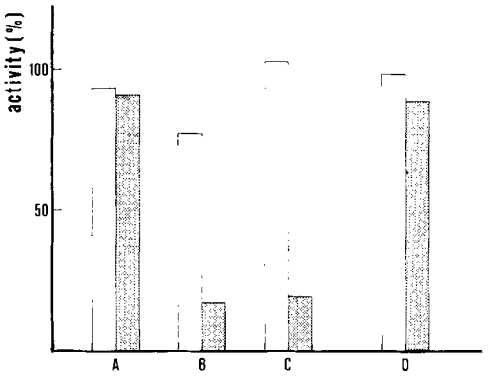


Fig. 7- 5'-nucleotidase and marker activities in white and resealed ghosts. Each activity is expressed as % of that of detergent-treated ghosts. The detergent treatment was made incubating the ghosts with 0.01% saponin(w/v, final concentration) for NADH-cytochrome *c* oxidoreductase activity determination, and with an equal volume of 0.2% TRITON X100(v/v) in all other cases. Each value represents average of 3 experiments. ☐ white ghosts; ▨ resealed ghosts. A=acetylcholinesterase; B=NADH-cytochrome *c* oxidoreductase; C=glyceraldehyde 3-phosphate dehydrogenase; D=5'-nucleotidase.

enzyme in leukocytes. Experiments carried out on hepatocytes(29) and adipocytes(30) led to the same conclusion.

Dornand et al.(2) showed the existence of a 5'-nucleotidase-facilitated adenosine transport in lymphocytes. As lymphocytes, erythrocytes do not carry out *ex novo* synthesis of purines(31), but exhibit turnover of adenine nucleotides(31,32). Then erythrocytes depend on the environment availibility of purine, particularly adenine derivatives. The presence and sidedness of a 5'-nucleotidase on the exterior aspect of plasma membrane might be related to adenosine supply for red cells. Experiments are in progress on red cells to verify this hypothesis.

Acknowledgements

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