# THE HETEROGENEITY OF ERYTHROCYTE IMP:PYROPHOSPHATE PHOSPHORIBOSYL TRANSFERASE AND PURINE NUCLEOSIDE PHOSPHORYLASE BY ISOELECTRIC FOCUSING

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## 1. Introduction

The recent demonstration that human erythrocyte IMP:pyrophosphate phosphoribosyl transferase (HxPRTase) has properties compatible with it being subject to allosteric regulation [1] has raised the possibility of the existence of subunits in this enzyme. The structural gene for HxPRTase is believed to be X-linked, but no variant of the human red cell enzyme was detected in 382 blood samples examined by electrophoresis in starch gel [2]. Four isoenzymes of HxPRTase have, however, been demonstrated by Bakay and Nyhan [3] using columns of polyacrylamide gel and a partially purified enzyme preparation from human erythrocytes. We wish to report here the results of some isoelectric focusing experiments which clearly demonstrate the heterogeneity of this enzyme in haemolysed human erythrocytes, and which also suggest an inter-relationship between this enzyme controlling the conversion of hypoxanthine to IMP and the enzyme purine nucleoside phosphorylase (NP) which converts hypoxanthine to inosine.

## 2. Materials and methods

Thin layer plates of polyacrylamide gel were prepared by photopolymerisation with 0.003% riboflavin and 7.5% "Cyanogum 41" (B.D.H., London) in deionised water containing 2% Ampholine carrier ampholites (L.K.B., Sweden). The gels were prepared and electrophoresed and the pH of sections determined essentially as described by Awdeh et al. [4] except that a Shandon electrophoresis tank (Model U77) was adapted for running the gels. The base of the tank, including the Pt electrodes, was covered with plastic foam material (1 cm thick) which served to maintain a moist atmosphere throughout the period of electrophoresis. The tensioning rods were removed and two carbon rods (0.6 X 26 cm) of type link M.S. (Ship Carbon Co., England) were supported in the hard rubber clips at the ends of the shoulder pieces. Crocodile clips were used to connect the rods to terminals of a VoKam constant voltage/constant current power supply. This simple adaption permitted isoelectric focusing of gels of varying length, allowing rapid reconversion of the tank for normal electrophoresis at any time.

Gels were run at 4° inverted over the carbon rods, with a narrow strip of moist filter paper between the gel and each rod to ensure uniform contact. After applying 200 V for 2 hr to pre-focus the ampholites, samples (10–20 μl) were pipetted onto 1 cm squares of filter paper and placed in contact with the gel 2 cm from the cathode end. Electrofocusing was carried out at 200 V (0.5–1.0 mA) per gel for 20–40 hr.

Enzyme proteins were located by applying an agar overlay (1.5% in 0.05 M Tris (hydroxymethyl-aminomethane) buffer pH 7.4) containing (8-$^{14}$C)-hypoxanthine (3 μM; specific activity 60.1 mCi/mmole, Amersham), $MgCl_2$ (5 mM) and the appropriate ribosyl donor i.e. 5-phosphoribosyl-1-pyrophosphate tetra Na salt (2 mM) for HxPRTase or α-D-ribose-1-phosphate dicyclohexyl ammonium salt (2 mM) for NP enzymes. After incubation at 37° for 1–2 hr, the agar overlay was removed, cut into strips 1 cm wide corresponding to each sample application, and each

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agar strip covered with moist DEAE (Whatman Grade DE 81) paper, good contact being maintained by light weighting between glass plates for ½ hr. DEAE papers were then subjected to descending chromatography for 1 hr in deionised water pH 5.0. Under these conditions nucleotide products remain at the area of contact of the paper with the gel, nucleosides are washed 2–4 cm down from the contact area and Hx is completely eluted. After drying, DEAE papers were cut into sections (0.5 X 0.5 cm) and their radioactivity measured in 10 ml toluene-containing scintillator Butyl PBD (0.5% w/v, Ciba) in a liquid scintillation spectrometer.

Radioautographs were taken from dried DEAE papers by placing them in contact with Kodirex X-ray film for approximately 3 days.

Samples run in a "Gradipore" (Universal Scientific Ltd.) were electrophoresed for 12 hr, at 100 V and 35 mA in 0.05 M Tris buffer containing 2.5 mM $MgCl_2$, and the enzyme proteins were located as in the isoelectric focusing experiments.

## 3. Results and discussion

Fig. 1 demonstrates the heterogeneity of the enzyme HxPRTase in freshly lysed erythrocytes from two normal subjects, and it can be seen that a similar profile is obtained by two different methods of lysing the cells.

Similar patterns of enzyme distribution have been obtained from the freshly lysed red cells of eight other humans and six pigs. Storage of haemolysates at −25° (3 days to 6 months) results in alteration of the enzyme profiles as illustrated for 3 subjects in fig. 2, where in all cases the peak at pI 6.0 now shows the greatest activity (cf. peak pI 6.5 prior to storage). When areas of polyacrylamide gel (1 X 1 cm) containing proteins precipitated at pI 7.5, 6.5 and 6.0 were refocused on a second gel under the same conditions, proteins pI 6.5 and 6.0 refocused as single peaks at the same pI values, respectively, whereas enzyme pI 7.5 showed no activity at its original pI value, but gave rise to peaks of approximately equal activity at pI 6.5 and 6.0. When subjected to "Gradipore" analysis proteins pI 6.5 and 6.0 were both located in a position corresponding to 12% gel whereas protein pI 7.5 was

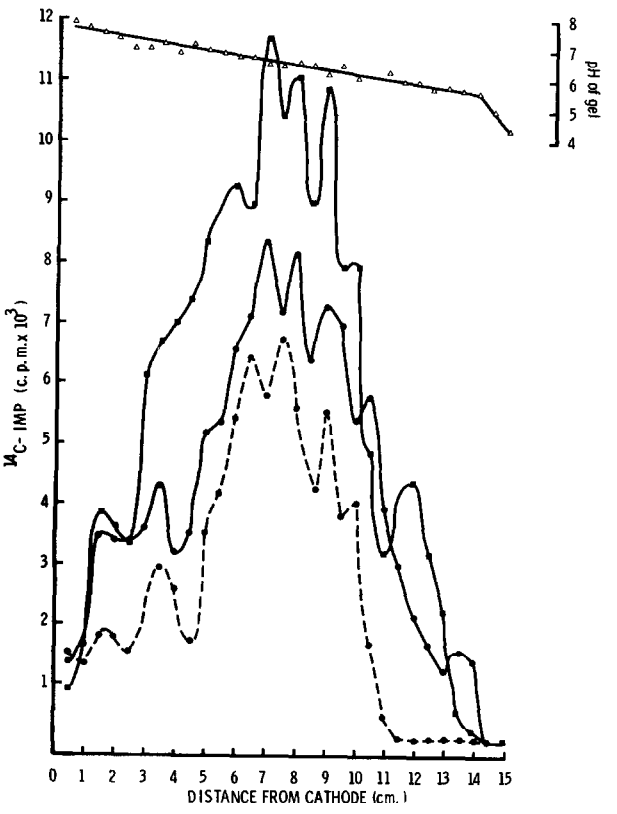


Fig. 1. Typical distribution of the HxPRTase isoenzymes shown by isoelectric focusing for 20 hr. Profiles (●——●), (■——■) were obtained from two normal subjects (A, B respectively) on erythrocytes lysed by rapid freezing (at −70°) and thawing three times and (●- - -●) refers to a sample from subject A lysed in an equal volume of 0.45% saline with centrifugation over $CCl_4$ for 50 min to remove cell debris.

retarded at 7.5% gel. These results suggest that proteins pI 6.5 and 6.0 are differently charged proteins of similar molecular weight and that both may have originated from the higher molecular weight protein pI 7.5.

A partially purified preparation of HxPRTase prepared from human haemolysates as described by Craft et al. [1] showed a distribution of isoenzymes by radioautography similar to those shown in fig. 2, but "Gradipore" analysis of this preparation indicated a molecular weight of the same order as that of haemoglobin (68,000). This value is in agreement with that of Der Kaloustian et al. [2] obtained from samples of

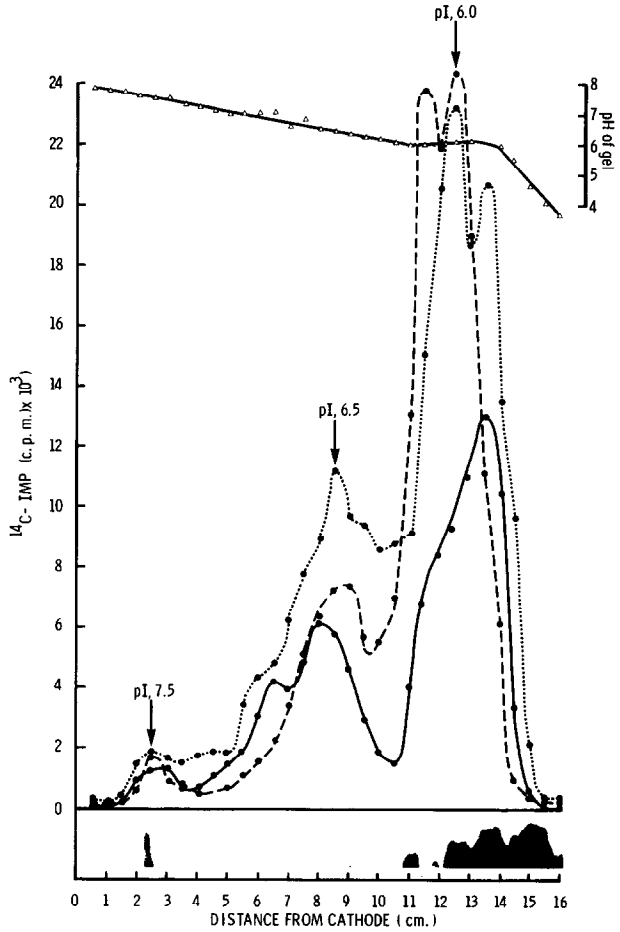


Fig. 2. Distribution of HxPRTase isoenzymes after 40 hr isoelectric focusing of haemolysates stored for 3 days at −20° prior to application. Profile (•·····•) refers to subject A and profiles (•——•) and (• - - - - •) were from two gout patients, known to have normal levels of the PRTase. The radioautograph below shows the distribution of NP isozymes for subject A under the same conditions of isoelectric focusing.

human, sheep, rabbit and chicken erythrocytes and also with the estimate of 60,000 (±10%) given for HxPRTase purified from human haemolysates (Krenitsky et al. [5]), but clearly this molecular weight value refers to the more stable protein subunits precipitated at pI 6.5 and 6.0.

Fig. 3 demonstrates a striking similarity between the profiles for HxPRTase and NP enzymes in the stored haemolysates of a gout patient R, whose intact erythrocytes have been shown to have one tenth of normal activity for HxPRTase, but normal activity for NP. Profiles with coincident peak activity for these two enzymes have also been observed in haemolysates of the three subjects whose HxPRTase isoenzymes are shown in fig. 2 and also in the partially purified preparation used in "Gradipore" analysis. In addition, a preparation of NP from calf spleen (Boehringer) gave enzyme peaks at pI 7.5, 6.5 and 6.0 when either PRPP or ribose-1-phosphate was added to $^{14}$C-hypoxanthine in the overlay. When ribose-1-phosphate is the ribosyl donor, less than 200 cpm are found in the contact areas of DEAE papers, indicating virtually no ribotide formation under the conditions for detecting NP enzymes. Using PRPP, however, although most of the $^{14}$C activity is located in the contact area as nucleotides, there is often in addition considerable $^{14}$C activity in

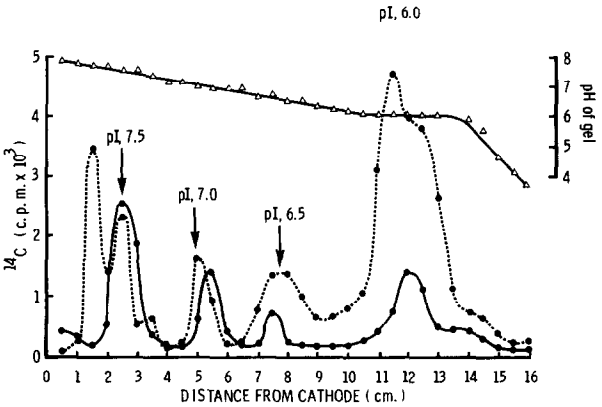


Fig. 3. Profiles (●——●) and (● - - - ●) show the distribution of HxPRTase and NP isoenzymes respectively after 40 hr isoelectric focusing of haemolysates from gout patient R, who is known to have a partial deficiency of HxPRTase.

the nucleoside area, with low background counts between the two products. The conditions used for chromatographing DEAE papers do not produce inosine from adsorbed IMP, so its formation can be accounted for either by 5′-nucleotidase activity of proteins adsorbed onto DEAE papers or by ribose phosphate which may be present as impurity or formed during the incubation from PRPP.

When hypoxanthine was replaced by adenine in the substrate overlay, a single narrow peak for $^{14}$C-AMP formation at pI 5.5 was obtained. Guanine, however produced peaks for $^{14}$C-GMP formation coincident with those for $^{14}$C-IMP formation in haemolysates from 3 humans and 1 pig. It has already been well established for both HxPRTase and NP from human erythrocytes that guanine, but not adenine can replace hypoxanthine as substrate.

The aggregate subunit nature of NP enzymes purified 600–800 fold from human erythrocytes has been demonstrated by Pinto [6] and Agarwal et al. [7] have demonstrated that there are at least 3 binding sites for $^{14}$C-hypoxanthine per molecule of a crystalline preparation of this enzyme purified from human erythrocytes. Edwards et al. [8] have suggested that a primary isozymic form of human NP is modified *in vivo* with generation of several secondary isozymes. These authors report a molecular weight of 84,000 for human NP by gel filtration chromatography and postulate that the enzyme has a trimeric structure.

The radioautograph shown under fig. 2 for NP isozymes of subject A clearly demonstrates that bands of greatest intensity are coincident with the peaks that correspond to HxPRTase in this subject (fig. 2). Further radioautography has confirmed that none of the peaks in the profiles of fig. 2 are due to a single enzyme protein, each peak representing a varying number of bands on the radioautograph.

All these observations suggest that activity for the two enzymes i.e. the sites of attachment for PRPP and ribose-1-phosphate are located in the same protein moieties. A comparison of figs. 2 and 3 shows that patient R has a normal level of activity for HxPRTase at pI 7.5, but considerably reduced activity notably at pI 6.0, but also at pI 6.5. Peak activity for his NP enzymes, however, were not appreciably lower than those for normal subjects. Differing affinities of the substrates PRPP and ribose-1-phosphate for active sites of sub-units pI 6.5 and 6.0 and the aggregate protein pI 7.5 could account for these findings. A genetic mutation resulting in reduced affinity for PRPP would not necessarily affect the affinity of the site for the smaller ribose phosphate molecule.

The marked heterogeneity of both HxPRTase and NP demonstrated here by isoelectric focusing and the abundant distribution of these enzymes in body tissues suggest an important role for their function in human metabolism.

## References

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