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SHORT COMMUNICATIONS

Ionic influence on human erythrocyte catechol-O-methyltransferase activity

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Several reports have described methods for the determination of catechol-O-methyltransferase (COMT; EC 2.1.1.6) activity in human erythrocytes [1–6]. In these assay techniques, different catechol substrates, different buffers and different concentrations of magnesium have been used.

As a part of a methodological study on human erythrocyte COMT we here report some of the differences between COMT assays in Tris–HCl and phosphate buffers. The influence of added alkali salts on COMT activity is also examined.

The substrate 3,4-dihydroxybenzoic acid (DBA) was bought from Sigma Chemical Company. Radioactively labelled *S*-adenosyl-methionine (^{14}C-methyl-SAM, sp. act. 52.6 mCi/mmole) was purchased from New England Nuclear. All scintillation equipment were from Packard Instruments. Isoamylalcohol came from Fluka and all other chemicals were manufactured by Merck, West Germany. The alkali salts were of suprapure grade and the other chemicals were of analytical grade.

The general procedure of the COMT assay was as follows. Venous blood was collected in heparinized tubes. The red blood cells were washed twice in cold isotonic sodium chloride and centrifuged at 1500 *g* for 10 min. The cells were stored at −85° until assayed. 150 μl lysed cells (20 per cent erythrocytes in distilled water) were mixed with 110 μl buffer, pH 7.6, containing various specified concentrations of ions and 0.5 mM DBA, final concentration. After preincubation for 5 minutes at 37° the reaction was started by adding 10 μl of ^{14}C-SAM (15 μM final concentration). The reaction was stopped after 15 minutes with 100 μl 1 M HCl. Blanks were made without DBA. The product was extracted into 5 ml water saturated toluene/isoamylalcohol (7:3) for 20 s on a Vortex mixer. The radioactivity in 4 ml of the organic phase was measured in a liquid scintillation counter (Packard 3380). The activity is expressed as nanomoles product formed per ml packed red blood cells per hour.

The concentrations of calcium and magnesium were determined with a Pye Unicam atomic absorption spectrophotometer (SP 192).

The relation between magnesium concentration and erythrocyte COMT activity from one individual in Tris–HCl and phosphate buffers of two concentrations is shown in Fig. 1. The maximal COMT activity was increased in phosphate buffer compared to Tris–HCl buffer. The optimal magnesium concentration was higher in phosphate buffer than in Tris buffer. A shift in the optimal magnesium concentration was also observed when the concentration of phosphate was increased. Any inhibitory effect of Tris on the COMT activity could be excluded as the addition of increasing amounts of Tris to an 0.08 M phosphate buffered system with 1 mM $MgCl_2$ had no effect. Instead the activity increased when phosphate was added to an 0.08 M Tris buffered system. There was no difference in apparent K_m values for DBA in the Tris and phosphate buffered solutions.

A difference between equimolar Tris–HCl and phosphate buffers was also seen when studying the calcium inhibition [4, 7, 8] of erythrocyte COMT activity (Fig. 2). The calcium ions exhibited a stronger inhibitory effect when Tris buffer rather than phosphate buffer was used. Optimal magnesium concentrations were used in both buffers. Without exter-

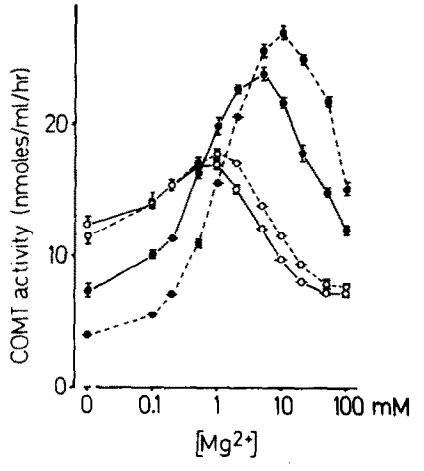

Fig. 1. The relation between added Mg^{2+} concentration and COMT activity in two different buffer systems (pH 7.6) of two concentrations, 0.08 M Tris–HCl (○——○), 0.16 M Tris–HCl (○- - -○), 0.08 M potassium phosphate (●——●) and 0.16 M potassium phosphate (●- - -●). Each point represents the mean of duplicates and the error bars represent the range of values.

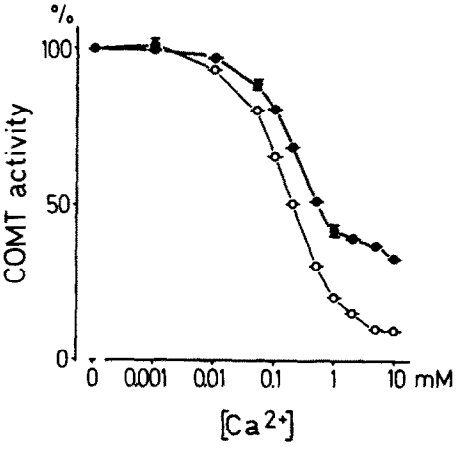

Fig. 2. The decrease in COMT activity after addition of increasing amounts of Ca^{2+} in 0.08 M phosphate buffer (●——●) and 0.08 M Tris–HCl buffer (○——○) pH 7.6. Each point represents the mean of duplicates and is expressed as percentage of the activity without Ca^{2+} added. The error bars represent the range of values.

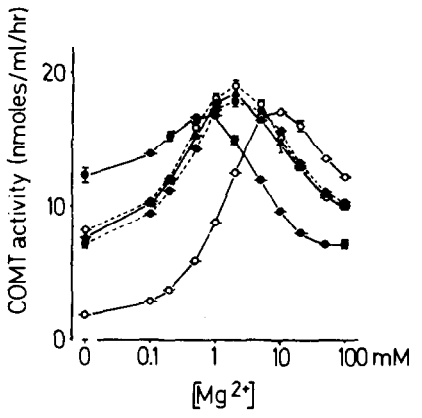

Fig. 3. Influence of Mg^{2+} concentration on COMT activity without salt added (●——●) and in presence of 0.3 M NaCl (○---○), 0.3 M KCl (▲——▲), 0.3 M RbCl (●---●) and 0.3 M LiCl (○——○) in 0.08 M Tris-HCl buffer, pH 7.6. Each point represents the mean of duplicates and the error bars represent the range of values.

nally added Ca^{2+}, the total calcium concentration was below 10 μM as measured by atomic absorption spectrophotometry.

The addition of 0.3 M NaCl, KCl or RbCl to a 0.08 M Tris-HCl buffer also altered the optimal magnesium concentration (Fig. 3). Inhibition of the COMT activity was observed at low magnesium concentrations (0–0.5 mM added $MgCl_2$). At higher concentrations of $MgCl_2$ (1–100 mM) sodium, potassium and rubidium chloride increased the COMT activity up to 15 per cent. The amount of extracted product was not altered if the salt was added after the incubation but prior to the extraction. Thus the salt effect was not caused by an altered extractability of the product. Although lithium ions exhibited a stronger inhibitory effect on the COMT activity than the other alkali metal ions (Fig. 3), the lithium inhibition could also be compensated by an increase in the magnesium concentration.

The use of phosphate buffer in the COMT assay caused a precipitation in the reaction tube. This has been described earlier [4] when the formation of a white precipitate, 'possibly calcium and/or magnesium phosphate' was observed when COMT was assayed in phosphate buffer. This interaction between magnesium and phosphate could be the cause of the higher magnesium optimum obtained in phosphate buffer compared to Tris-HCl buffer (Fig. 1). Higher concentrations would be needed in phosphate buffer to obtain enough free magnesium ions for the enzyme reaction. Also the decreased COMT activity in phosphate buffer compared to Tris-HCl buffer at low magnesium concentrations (below 0.5 mM $MgCl_2$) could be due to the magnesium phosphate interaction. The difference in calcium inhibition between the two buffers (Fig. 2) could be explained in the same way because phosphate forms a complex also with calcium ions and therefore leaves less free calcium ions to inhibit the enzyme.

Apart from the formation of complex with magnesium, equimolar Tris-HCl and phosphate buffers differ also in ionic strength. At pH 7.6 the ionic strength in a phosphate buffer is about three times higher than in an equimolar Tris-HCl buffer. Increased ionic strength seems to increase the COMT activity since doubling the buffer concentration caused a higher maximum activity (Fig. 1). However, the higher ionic strength in the phosphate buffer than in the Tris buffer is not enough to explain the 50 per cent increase in maximal COMT activity since adding salt (e.g. NaCl) to the Tris buffer up to the same ionic strength as in 0.16 M phosphate buffer increased the maximum activity only up to 15 per cent (Fig. 3). Whether the remaining increase in COMT activity was due to an activation by the phosphate ions or some other molecular interaction in the assay system could not be concluded from the present results.

All the different alkali metal ions added to the buffer in these experiments seemed to compete with the magnesium activation of COMT. Lithium differed from the other alkali metal ions in that a higher magnesium concentration was needed to overcome the inhibitory effect (fig. 3). Lithium has earlier been shown to inhibit other magnesium dependent enzymes [9]. The inhibitory effect is probably due to the similarities in atomic radius and crystal ionic radius between the two elements [9].

These experiments show some of the effects of using different ionic compositions of the incubation medium for human erythrocyte COMT assays. However, further experiments are needed to elucidate the kinetic and molecular mechanism of these effects.

In conclusion, the dependency of human erythrocyte COMT activity on Mg^{2+} concentration was found to be different in phosphate and in Tris-HCl buffer. Higher magnesium concentrations were required in phosphate buffer to obtain maximal COMT activity. The maximal COMT activity was higher in phosphate than in Tris-HCl buffer. In contrast the COMT activity was lower in phosphate buffer at low magnesium concentrations. NaCl, KCl, RbCl and LiCl in the incubate also changed the magnesium dependency of the COMT activity and inhibited the enzyme at low Mg^{2+} concentrations. LiCl differed from the other alkali salts in that a higher magnesium concentration was needed to overcome the inhibitory effect.

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