4-Aminobutyrate aminotransferase (EC 2.6.1.19) from P. aeruginosa PAO1 which also transaminates acetylornithine and ornithine was purified to electrophoretic homogeneity as judged by gel electrophoresis[7]. ACOAT and 4-aminobutyrate aminotransferase show similar catalytic properties. It was therefore interesting to determine whether the 2 enzymes have a common subunit or at least related surface structures. No cross-reactivity with 4-aminobutyrate aminotransferase could be detected.

Precipitates of ACOAT with anti-ACOAT were formed by incubating 20 µl of mouse anti-ACOAT antiserum with 130 µl of phosphate buffered saline and 50 µl of enzyme solution containing 25 µg of ACOAT. 63% of the original enzyme activity was found in the precipitate while 2% remained in the supernatant. This result resembles the one found for N-acetylglutamate 5-phosphotransferase from the same organism[3]. Enzymatic activity was assayed as described previously[4].

Another interesting finding resulted from experiments using Ouchterlony double diffusion. Purified ACOAT and ACOAT from a crude extract run alongside displayed a pattern of partial identity with a spur forming towards the well of the crude preparation (figure 2). This observation indicates that the structure of the enzyme might have been changed somewhat during the purification. Such an alteration could be caused by the loss of a ligand or of the cofactor.

ACOAT appears to be induced by arginine and to be repressed by various carbon sources[4]. In order to understand the regulation of the enzyme, it was crucial to determine whether the control is exerted at the level of enzyme activity or of enzyme synthesis. The radial monodiffusion technique described by Mancini[8] offered a convenient means to measure the concentration of a specific protein, not depending on the catalytic activity. Mancini assay confirmed the induction of enzyme synthesis by arginine and its repression by different carbon sources. The 2% agar gels contained 2% of antiserum. The relative content of ACOAT of an extract from strain PAO1 grown on mineral salts medium with arginine as the only source of carbon and nitrogen was 100. The same strain cultivated with arginine and citrate revealed a relative enzyme content of 21, while the amount of enzyme in uninduced cells grown on glutamate as the sole C and N source was 6. Thus the enzyme is induced by arginine at least 16–17fold. The data obtained by this approach agree very well with measurements of enzyme activity.

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The effect of pyrophosphate and diphosphonates on calcium transport in red cells

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Summary. The effect of 0.5 mM pyrophosphate (PPi), disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethane diphosphonate (Cl_2MDP) on the ATP-dependent Ca^{2+} extrusion from the human red cell ghosts was studied. PPi and Cl_2MDP had no effect, when introduced into the cells or added outside to the medium. EHDP slightly increased the calcium concentration in the released cells and slightly decreased the rate constant of the calcium transport, having opposite effects when it was inside or outside the cells. PPi and the 2 diphosphonates were not found to move easily across the red cell membrane.

Pyrophosphate (PPi[2]), a product of many biosynthetic reactions[3], inhibits apatite formation[4] and dissolution[5]; and it has therefore been suggested to regulate bone turnover[5]. The diphosphonates EHDP[2] and Cl_2MDP[2] are compounds structurally related to PPi, containing a P-C-P instead of a P-O-P bond. In contrast to PPi, they are resistant to hydrolysis and are more potent in vivo than PPi. They are powerful inhibitors of ectopic calcification[6], bone calcification[7] and bone resorption[7,8]. These effects might be explained by the action on crystal

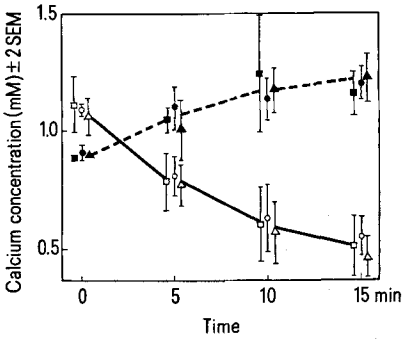

Fig. 1. Ca^{2+} transport of human red cells in the presence of 0.5 mM Cl_2MDP. Open signs represent Ca^{2+} concentration in the cells; filled signs represent Ca^{2+} concentration in the incubation medium; control (○, ●); Cl_2MDP within the cells (□, ■); Cl_2MDP in the incubation medium (△, ▲). Values are given ± 2 SEM.

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formation and dissolution, but it is concievable that cellular effects are also involved. Indeed there is no good correlation between the effect on crystal dissolution and the inhibition of bone resorption[8]. There is also evidence of an active renal transport of diphosphonates, which suggests an active cellular uptake of these compounds[9]. Recently it was observed that the diphosphonates inhibit the lactate production in cultured cells[10]. Actually both diphosphonates have been found to retard the release of calcium from mitochondria when added in vitro[11] or in vivo[12]. Furthermore PPi has been found to increase the influx of calcium into kidney cells[13], and to stimulate at low concentrations the calcium uptake into embryonic bone cultivated in tissue culture medium[14].

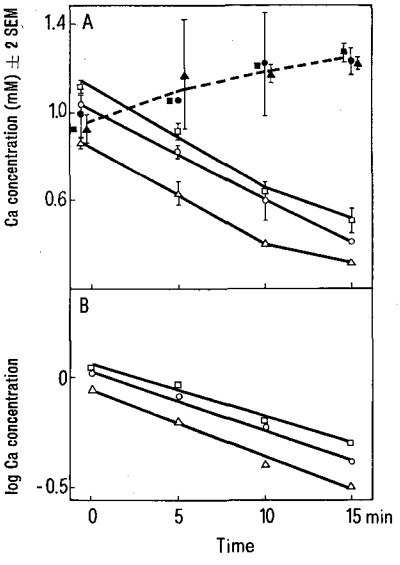

Fig. 2. Ca^{2+} transport of human red cells in the presence of 0.5 mM EHDP. Open signs represent Ca^{2+} concentration in the cells; filled signs represent Ca^{2+} concentration in the incubation medium; control (○, ●); EHDP inside (□, ■); EHDP outside (△, ▲). Values are given ± 2 SEM. When the SEM were smaller than these symbols, they were not included. In figure 2 B the calcium concentration in the blood cells is represented on a logarithmic scale.

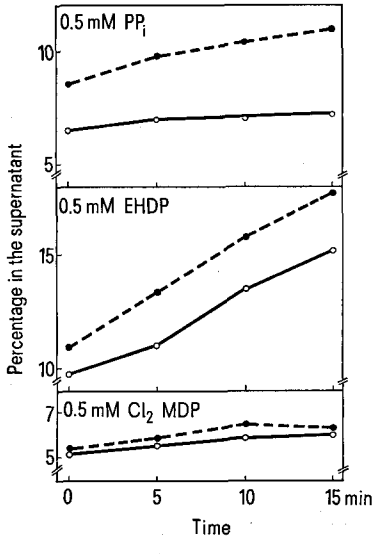

Fig. 3. Permeability of red cell membrane for PPi, EHDP and Cl_2MDP. Ghosts which had been filled with ^{32}P-PPi, ^{14}C-EHDP and ^{14}C-Cl_2MDP were incubated and the radioactivity measured in the medium and in the cells. The amount found in the medium is expressed as percentage of the amount found in the cells. ●– –●, radioactivity; ○—○ hemoglobin as control.

It is possible that the effects of PPi and the diphosphonates are due to an effect on the active transport of calcium through the cell membrane. To test this possibility, a simple system was chosen, namely the calcium transport in human red cells, a system which has been studied throughout[15]. The effect of PPi, EHDP and Cl_2MDP in this system is described in this communication.

Materials and methods. EHDP, Cl_2MDP, ^{14}C-EHDP and ^{14}C-Cl_2MDP were obtained from the Procter and Gamble Company, Cincinnati (Ohio 45239, USA), ^{32}P-PPi from New England Nuclear, Dreieichenhain (Fed. Rep. of Germany), and all the other reagents (analytical grade) from Merck, Darmstadt (Fed. Rep. of Germany).

The experiments were carried out as described by Schatzmann and Vincenzi[15]. The red blood cells (obtained from the blood bank of the Swiss Red Cross) were washed 5 times with a 5 fold volume of isotonic salt solution buffered with 0.01 M imidazole-HCl, pH 7.2. The buffy coat was aspirated.

Ca^{2+}, Mg^{2+}, ATP and other reagents were introduced into the cells by reversal of hemolysis. At room temperature 6 ml of washed red blood cells were hemolysed in 25 ml of a solution containing 0.75 mM $CaCl_2$, 4 mM $MgCl_2$, 2 mM ATP, 5 mM imidazole-HCl, the pH of the solution was adjusted to pH 7.2, once PPi, EHDP or Cl_2MDP were added, $MgCl_2$ was increased to 4.5 mM to correct for possible binding. After 135 sec, the cells were resealed by adding 1.315 ml 3 M KCl and, after additional 3 min, the cell suspension was diluted with 48 ml of an ice-cold solution containing 0.11 M KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$ and 40 mM imidazole-HCl, pH 7.2. The cells were then spun at 10,000 × g for 5 min in a refrigerated centrifuge. The pellet was then resuspended and the cells rewashed twice more, after which a suspension was prepared with a haematocrit between 0.3 and 0.4. When necessary PPi, EHDP or Cl_2MDP were added to the final cell suspension. The cells preparation (15 ml) was then incubated in 25 ml Erlenmeyer flasks in a shaking water-bath at 25 °C. Samples were taken at time 0, 5, 10 and 15 min, immediately cooled in ice-water and centrifuged at 24,000 × g for 2 min (acceleration time included). The haematocrit was determined before and after the incubation.

The pellet was diluted 1:3 and the supernatant 1:2 with water. Both solutions were then deprotonized with 10% trichloroacetic acid containing 2% (w/v) $LaCl_2$. Calcium was determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 290 B.

To investigate whether PPi, EHDP of Cl_2MDP can move across the red cell membrane, ^{32}P-PPi, ^{14}C-EHDP, or ^{14}C-Cl_2MDP (final specific activity: 0.053; 0.1; 0.24 μCi/

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μmole respectively) were added to the solution used to hemolyse the cells. After the incubation, the radioactivity of cells and medium was determined in the supernatant after precipitation of membranes and proteins with trichloroacetic acid, using a Packard Tri-Carb liquid scintillation spectrometer, Model 3324. The leaking of the cells was estimated by measuring hemoglobin in the cells and the medium[16].

Results and discussion. Figure 1 shows the ATP driven calcium transport out of the sealed ghosts. As found by others[15], the calcium concentration inside the cells is higher than in the hemolysing solution added before resealing. This effect is probably due both to the shrinking of the ghosts upon reversal of hemolysis and some binding of calcium[17]. Cl_2MDP showed no effects, whether it was present inside or outside the cells, PPi did not produce any effect either (not shown). It is unlikely that PPi was hydrolysed during the experiment, since calcium is a strong inhibitor of inorganic pyrophosphate[18,19]. Actually we found that not more than 10% of the PPi was hydrolysed, when given at 1 mM concentration in the absence of ATP. Addition of ATP will not bind enough calcium to abolish the inhibitory effect of the latter.

Results are somewhat different for EHDP (figure 2). When EHDP was introduced into the cells, calcium concentrations found inside the cell at time 0 was higher than in the control experiment. The opposite was the case, when EHDP was added outside into the incubation medium. To analyse a possible effect on the rate of transport, assuming a first order reaction, the calcium concentrations within the cells were plotted on a logarithmic scale versus time. The following rate constants were found ($\pm$ 1 SEM): 0.0622 $\pm$ 0.0023 min^{-1} for control; 0.0522 $\pm$ 0.0035 min^{-1} for EHDP inside and 0.0693 $\pm$ 0.0032 min^{-1} for EHDP outside. The latter 2 values are significantly different ($p < 0.025$), suggesting that EHDP has a slight effect on the calcium transport out of the cell. It is possible that EHDP may bind to the cell membrane, where it may act differently on the transport whether it is outside or inside. The small difference in the rate constants could explain the difference of the Ca^{2+} content of the cells at time 0, when EHDP was present, since the calcium transport is probably not completely stopped during the time from sealing of the cells up to the beginning of the experiment, even though the cells were kept in ice. The difference in the calcium content could also be explained in that EHDP may influence the shrinking of the ghosts or possibly the binding of Ca^{2+} to the membrane. Furthermore one could argue that EHDP binds Ca^{2+} and that the transport is changed because the free Ca^{2+} concentration is decreased. This, however, is very unlikely since PPi and Cl_2MDP have a similar affinity for Ca^{2+} as does EHDP[20].

When ghosts containing labelled PPi or labelled diphosphonates were incubated, a loss of these compounds was found which was only slightly faster than hemoglobin in the case of PPi and EHDP and was the same as hemoglobin in the case of Cl_2MDP (figure 3). Thus is appears that PPi and the diphosphonates cannot easily permeate the erythrocyte membrane.

In conclusion, it seems that PPi and the 2 diphosphonates EHDP and Cl_2MDP have no striking effect on the calcium transport of red blood cells and that these compounds do not easily permeate the erythrocyte membrane.

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Influence of protein free aqueous extract of parathyroid powder on serum vitamin A level in rats

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Summary. Administration of the protein-free extract of bovine parathyroid powder to rats resulted in a significant increase of the serum vitamin A level.

The oral administration of bovine parathyroid powder, as well as its protein-free aqueous extract, showed widerange vitamin A-like effects[2]. This observation prompted us to test whether or not the protein-free and calcium-inactive (consequently parathormone-free) extract of the powder has any influence on serum vitamin A level in rats.

Preparation of protein-free parathyroid extract. Freeze-dried and micronized bovine parathyroid glands (Biofac A/S, Copenhagen) were defatted according to Aurbach[3]. Eventual presence of thyroid hormones in the powder was excluded by the organically bound iodine assay of US Pharmacopoeia[4]. 10 g of the defatted powder was successively stirred for 1 h with 3 × 100 ml of distilled water at 30 °C. The combined supernatant separated by centrifugation was freeze-dried. The residue was dissolved in 20 ml of water, then 8 ml of 60% (w/v) aqueous solution of trichloroacetic acid was added dropwise, with stirring, and left to stand for 2 h at 5 °C. The aqueous supernatant separated by centrifugation from precipitated proteins was extracted with 20 × 120 ml of ether. Traces of ether were removed by evaporation at reduced pressure at 25 °C, then the aqueous solution was freeze-dried to yield 1.2 g of dry protein free extract.

Effect of protein-parathyroid extract. 9 groups of male Wistar rats (LATI, Gödöllö, 200–220 g b.wt) were treated with different daily doses of the protein-free extract, the first one serving as control group. Each group consisted of 20 animals. Each animal was kept in a separate cage and received 20–25 g of feed daily. The standard feed contained 6 IU of vitamin A per g. The protein-free extract was administered on 8 consecutive days in 2 ml drinking water. On the 9th day, the rats were killed by decapitation. The vitamin A concentration in the serum