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INHIBITION BY LEAD OF HUMAN ERYTHROCYTE ($Na^+ + K^+$)-ADENOSINE TRIPHOSPHATASE ASSOCIATED WITH BINDING OF ^{210}Pb TO MEMBRANE FRAGMENTS *

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Summary

Fragmented human erythrocyte membranes were exposed to $PbCl_2$ for 10—40 min at 23°C prior to ($Na^+ + K^+$)-ATPase assay. Inhibition increased with exposure time. Enzyme activity in 5 μg membrane protein was inhibited 50% after a 10-min exposure to 1.0 nmol $PbCl_2$ (25 μM final concentration) and was inhibited 100% after 40 min. When membranes at various concentrations were exposed to $PbCl_2$ for 40 min, inhibition was linear with the ratio of $PbCl_2$ to protein. Inhibition of 100% was obtained at 0.2 nmol $PbCl_2$/μg protein. A graph of activity vs. [protein] in the presence of $PbCl_2$ intercepted the abscissa to the right of the origin, indicating that lead acts as an irreversible or very slowly reversible inhibitor. Addition of 1 mM 2,3-dimercaptopropanol, 1,3-dithiothreitol, DL-penicillamine or EDTA after 40 min exposure to 100 μM $PbCl_2$ restored 45, 64, 81 and 92% of the ($Na^+ + K^+$)-ATPase, respectively. These chelators, excluding EDTA, prevented inhibition when added before $PbCl_2$. Two washings of the membrane fragments with water or 10 mM imidazole-HCl (pH 7.4) did not restore activity. ^{210}Pb bound tightly to membrane fragments and beginning of saturation was observed at 0.19 nmol Pb^{2+} bound/μg protein. This corresponded to 200 μM final concentration of $PbCl_2$ in the ATPase assay. At 0.2 nmol $PbCl_2$/μg protein (100% inhibition of ATPase), from 0.10—0.17 nmol of lead was bound per μg protein. Under the same conditions, 1 mM DL-penicillamine removed 80% of the bound lead which correlated with its restoration of ATPase activity. Pb^{2+} does not appear to

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denature the enzyme. The irreversible kinetics may be related to sequestration of Pb^{2+} within vesicles that interfere with the accessibility of chelators to Pb^{2+} binding sites.

Introduction

Among the clinical symptoms of inorganic lead poisoning is anemia. Possible molecular mechanisms which have been suggested include defects in heme synthesis [1,2] and direct effects of lead on the erythrocyte membrane. Lead binding to red cells in vitro results in altered fragility [3] and ion transport [4,5]. Also, lead has been shown to induce conformational changes in the membrane proteins [6]. Deficiencies in the erythrocyte membrane (Na^+ + K^+)-ATPase have been reported in factory workers exposed to lead [7] and in urban children [8].

Inorganic lead has been found to inhibit the microsomal (Na^+ + K^+)-ATPase of rat brain, rat kidney [9] and *Electrophorus* electroplax [10]. In electroplax, the enzyme is inhibited non-competitively and reversibly but the extent of inhibition depends on the ratio of $PbCl_2$ to protein. The activity can be restored readily by dilution of the microsomes or by the addition of metal ion chelators. Similar results were found using preparations from human kidney and various organs of the dog [11].

In the present study, lead inhibition of human erythrocyte membrane (Na^+ + K^+)-ATPase is found to be irreversible or very slowly reversible in the absence of chelators. The inhibition can be correlated with binding of ^{210}Pb to the membranes.

Materials and Methods

Packed human red blood cells were obtained from the University of Michigan Blood Bank and the Southeastern Michigan Blood Center of the Red Cross. The Tris salt of ATP, DL-penicillamine, EDTA, 2,3-dimercaptopropanol and 1,3-dithiothreitol were obtained from Sigma Chemical Co. ICN and Amersham were the sources of [γ-^{32}P]ATP and $^{210}PbNO_3$, respectively. Enzyme incubations were performed in disposable borosilicate glass tubes (Kimble, No. 7300, 6 × 50 mm).

Red cell membranes were prepared according to the method of Post et al. [12] with certain modifications. Whole blood, 5 ml, was suspended in 0.5 ml of 0.2 M Na_4EDTA and 30 ml of 0.15 M NaCl. The cells were packed by centrifugation at 9750 × *g* for 30 min and were washed three more times in saline. Cell lysis was performed with 5 mM Tris-HCl (pH 7.4) and the fragments were sedimented at 9750 × *g* and washed twice more in this buffer. At this stage the pellets were pink. Further purification was obtained by resuspension in 4-ml of 0.1 M Tris-glycylglycine (pH 8.0) plus 25-ml water. If necessary, another wash in 0.5 mM Tris-glycylglycine (pH 8.0) was performed. All procedures were carried out at 0—2°C. The membranes were stored in a concentration of 8—12 mg protein/ml in 5 mM Tris-HCl (pH 7.4) at 4°C and were used within 2 weeks. Protein concentration measurements

were made according to the method of Lowry et al. [13]. The yield from this procedure averaged 16 mg membrane protein per 5 ml whole blood.

Hydrolysis of [γ-^{32}P]ATP was measured as described by Siegel and Albers [14]. The final reaction mixture contained 2 mM $MgCl_2$, 2 mM Tris-ATP, 10 mM imidazole-HCl (pH 7.4), 80 mM NaCl, 20 mM KCl, and membrane fragments containing 5—20 μg protein in a total volume of 40 μl. Mg^{2+}-ATPase activity was measured under all conditions, tested in the absence of NaCl and KCl and subtracted from the total to give (Na^+ + K^+)-increments. The enzyme was exposed to $PbCl_2$ for periods of 10—40 min at 23°C prior to assay. The activity of the control samples not exposed to lead was unaffected over these time periods. ATP hydrolysis was initiated by the addition of the reaction mixture. Incubations were carried out for 45 min at 37°C. The data represent the mean of duplicate samples that differ by less than 5%.

Dilution experiments were performed as follows. Membranes were exposed to $PbCl_2$ at a ratio of 0.2 nmol $PbCl_2/\mu g$ protein in a final volume of 375 μl at 23°C for 40 min. Five aliquots of 15 μl were removed for protein determination and ATPase assays. The remaining suspension was diluted by the addition of 3-ml H_2O or 10 mM imidazole-HCl (pH 7.4) and after 5 min at 23°C, was centrifuged at 12100 $\times g$ for 30 min. The supernatant portion was aspirated and the pellet was resuspended in 300-μl H_2O or buffer. Aliquots were removed and the procedure was repeated.

In the lead-binding experiments, erythrocyte membranes (0.5 mg protein) were exposed to 10—400 nmol $PbCl_2$ plus 30 pmol ^{210}Pb in a final volume of 375 μl (0.02—0.8 nmol $PbCl_2/\mu g$ protein). Aliquots were removed for scintillation counting (40-μl) and protein determination (15-μl). Following a 40 min exposure at 23°C the membranes were washed twice with imidazole-HCl exactly as in the dilution experiments for reversal of inhibition described above. The final pellets were resuspended in 225-μl of imidazole-HCl and aliquots were removed for measurements of protein and bound ^{210}Pb. ^{210}Pb determination was performed with the use of a Packard Model 3375 liquid scintillation spectrometer. The fluid consisted of 8 g 2,5-diphenyloxazole, 0.2 g 1,4-bis(2-(5-phenyloxazolyl))benzene, 1 l of Cellosolve * (purified). Parallel samples of incubation mixtures without membranes and subjected to the same washing procedures showed no significant retention of radioactivity by the glassware. In some of the lead experiments, DL-penicillamine was added after the 40 min exposure of protein to ^{210}Pb. After a second 40 min incubation at 23°C, the washing procedures were performed as described above.

Results and Discussion

Effects of Pb^{2+} on (Na^+ + K^+)-ATPase

Inhibition by $PbCl_2$ increases as a function of exposure time. When 5 μg membrane protein are first treated with 1 nmol $PbCl_2$ (25 μM), the (Na^+ + K^+)-ATPase activity is inhibited 50% at 10 min and 100% at 40 min of exposure (Fig. 1). The exposure time necessary to produce 100% inhibition varies with

* Cellosolve, ethyleneglycol monoethyl ether.

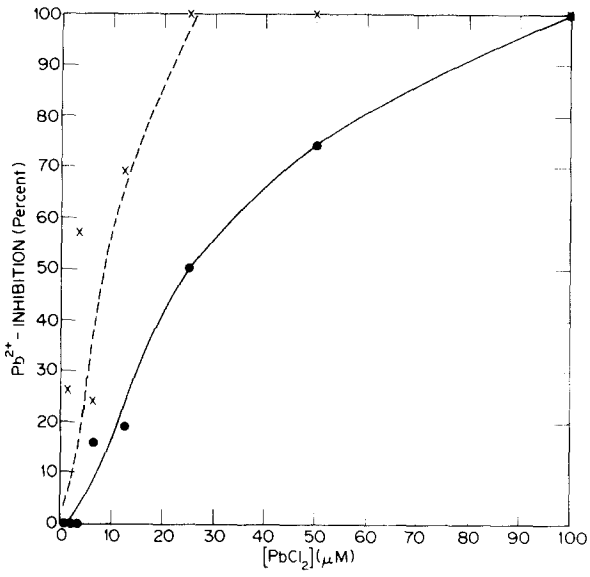

Fig. 1. $PbCl_2$ inhibition of erythrocyte ($Na^+ + K^+$)-ATPase at different exposure times. Conditions are described in Materials and Methods. Protein content is 5 μg/assay. The abscissa shows the total $PbCl_2$ concentration. Values are percent inhibition relative to controls exposed under the same conditions but without lead. ●———●, 10 min exposure to $PbCl_2$; (x- - - - -x), 40 min exposure to $PbCl_2$.

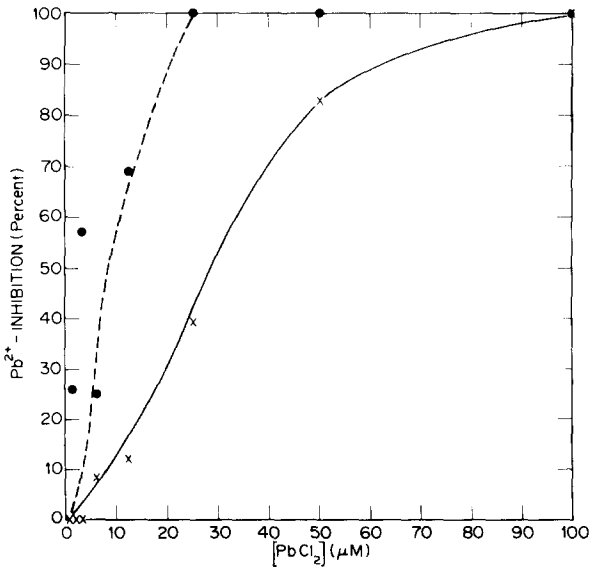

Fig. 2. $PbCl_2$ inhibition of erythrocyte ($Na^+ + K^+$)-ATPase at different protein concentrations. Conditions are described in Materials and Methods. A 40 min exposure of membranes to $PbCl_2$ preceded the assay. The abscissa shows the total $PbCl_2$ concentration. Values are percent inhibition relative to control samples exposed under the same conditions but without lead. ●- - - - -●, 5 μg protein/assay; x———x, 20 μg protein/assay.

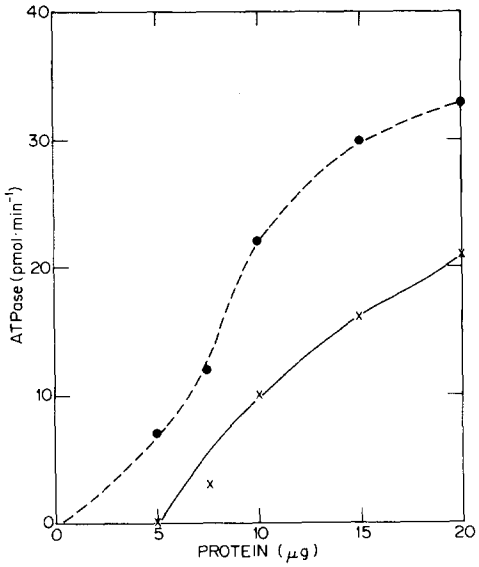

Fig. 3. Rate of ($Na^+ + K^+$)-dependent ATPase hydrolysis as a function of membrane protein concentration. Prior to assay the membrane fragments were exposed for 40 min at 23°C to H_2O (•- - - - - -•), or 1 nmol $PbCl_2$ (x———x) in 15 μl. The final concentration of $PbCl_2$ is 25 μM in a final volume of 40 μl after addition of reaction mixture.

protein concentration. At $PbCl_2$ concentration of 100 μM, enzyme activity in 5 μg protein is totally inhibited after an exposure time of 10 min (Fig. 1), but when 20 μg protein are employed 40 min of exposure are required for complete inhibition (Fig. 2). After 40 min exposure, the $[PbCl_2]_{0.5}$ is 8 and 28.5 μM for 5 and 20 μg protein, respectively (Fig. 2). The Mg^{2+}-ATPase activity is also inhibited by $PbCl_2$ and values for each data point have been subtracted to give the ($Na^+ + K^+$)-ATPase increments. The Mg^{2+} and ($Na^+ + K^+$)-ATPase activities of controls which were exposed to water rather than $PbCl_2$ were unaffected during these exposure conditions.

Inhibition of the ($Na^+ + K^+$)-ATPase is linear with the $PbCl_2$: protein ratio in the range from 0.05 to 0.2 nmol $PbCl_2$/μg protein (data not shown). The value for $[PbCl_2]_{0.5}$ corresponds to 0.085 nmol $PbCl_2$/μg protein at a time of exposure close to equilibrium. This is in agreement with values for half-maximal inhibition calculated from Fig. 2: 0.07 and 0.06 nmol $PbCl_2$/μg protein obtained with either 5 or 20 μg protein, respectively. Accurate data are difficult to obtain at low $PbCl_2$: protein ratios presumably because the excess amount of protein increases the number of non-enzyme binding sites and decreases free Pb^{2+} and also because of the difficulty in obtaining equilibrium at low concentrations.

After a 40 min exposure to 1 nmol $PbCl_2$ (25 μM), the rate of ATP hydrolysis is completely inhibited with 5 μg protein (Fig. 3). The fact that the curve intercepts the abscissa to the right of the origin suggests that the lead-induced inhibition is irreversible or very slowly reversible [15].

Washing experiments were performed in an attempt to dissociate the Pb^{2+} by dilution (Table I). Enzyme activity could not be restored by dilution

TABLE I

RESIDUAL Pb^{2+} INHIBITION OF ERYTHROCYTE (Na^+ + K^+)-ATPase AFTER WASHES

Membrane fragments containing 0.5 mg protein were exposed to 100 nmol $PbCl_2$ in a volume of 375 μl for 40 min at 23°C. Washing procedures are described in Materials and Methods. Values are percent inhibition of (Na^+ + K^+)-ATPase activity relative to control samples exposed under the same conditions but without lead and represent the mean of duplicate experiments that differ by less than 10%.

Treatment	% inhibition	
	H_2O washes	Imidazole-HCl washes
Initial	88	60
Wash 1	88	100
Wash 2	100	—

either with water or imidazole-HCl buffer (pH 7.4). The dilution factors in this experiment were 200-fold for wash 1 and 40 000-fold for wash 2, assuming that the pellet volume was 15 μl after each centrifugation. A third wash also failed to reverse the Pb^{2+} inhibition; however 86% of the enzyme activity in the control samples was lost at this step.

Chelators, on the other hand, are able to partially reverse the inhibition of the ATPase by $PbCl_2$ when they are added after enzyme exposure to $PbCl_2$ (Table IIA). The chelators were added to the inhibited enzyme in 10-fold molar excess over the $PbCl_2$ for 40 min prior to the ATPase assay. The order of effectiveness in restoring activity after $PbCl_2$ inhibition is EDTA > DL-penicillamine > 1,3-dithiothreitol > 2,3-dimercaptopropanol. The reaction mixture for the enzyme incubated with EDTA contained 4 mM $MgCl_2$ instead of the 2 mM used in other assays because at 2 mM $MgCl_2$, EDTA reduces the activity of the controls by chelation of the Mg^{2+} needed for enzyme activation. In contrast to their partial restorative effects when added after inhibition has developed, penicillamine, dithiothreitol, and 2,3-dimercaptopropanol are able to completely prevent $PbCl_2$ inhibition of the (Na^+ + K^+)-ATPase

TABLE II

REVERSAL OF Pb^{2+} INHIBITION BY CHELATORS

(A) Membrane fragments containing 20 μg protein were exposed to 4.0 nmol $PbCl_2$ in a volume of 15 μl for 40 min at 23°C. Then, after adding 40 nmol chelator or an equivalent volume of water, membranes remained at 23°C for a second 40 min exposure. Values are percent inhibition relative to control samples treated with chelator but no $PbCl_2$. In both A and B, the reaction mixture for the samples receiving EDTA contained 4 mM $MgCl_2$. Otherwise, assays were performed as described in Materials and Methods. (B) The water or chelator was added to the membrane fragments 40 min prior to addition of $PbCl_2$. Values are percent inhibition relative to control samples treated in a similar fashion but without $PbCl_2$.

Chelator	% Inhibition	
	A	B
None	100	100
2,3-Dimercaptopropanol	55	0
1,3-Dithiothreitol	36	0
DL-Penicillamine	19	0
EDTA	8	20

when they are added to enzyme prior to $PbCl_2$ (Table II). The reduced ability of these chelators to restore activity might be due either to reduced accessibility to membrane-bound lead or to irreversible effects produced by lead. The reason for the 20% inhibition in the presence of EDTA in this experiment is not known.

Pb^{2+} binding to membrane fragments

We wished to see if the inhibitory effects of $PbCl_2$ on the (Na^+ + K^+)-ATPase could be correlated with Pb^{2+} binding to membrane fragments. We chose conditions of incubation time (40 min) and lead: protein ratios which correspond to conditions in Fig. 2. It was found that ^{210}Pb binds tightly to the membrane fragments and withstands at least two washings. Under the enzyme assay conditions, total inhibition of the (Na^+ + K^+)-ATPase occurs at 0.2 nmol $PbCl_2/\mu g$ protein. At this ratio, saturation of all binding sites has not occurred (Fig. 4) suggesting that Pb^{2+} is binding to non-enzyme sites on the membrane. At higher concentrations of $PbCl_2$, measurements of ^{210}Pb binding are impossible because of obvious clumping of the membranes.

DL-Penicillamine is able to remove 80% of bound Pb^{2+} when added in 10-fold excess 40 min after $PbCl_2$ exposure (0.2 nmol $PbCl_2/\mu g$ protein, data not shown). This observation is correlated with the ability of DL-penicillamine to restore 80% of the (Na^+ + K^+)-ATPase activity under the same conditions (Table IIA). This indicates that the Pb^{2+} does not irreversibly denature the enzyme and that enzyme inhibition depends on continued binding of Pb^{2+} to critical sites.

It is possible that lead may be sequestered inside small vesicles formed by

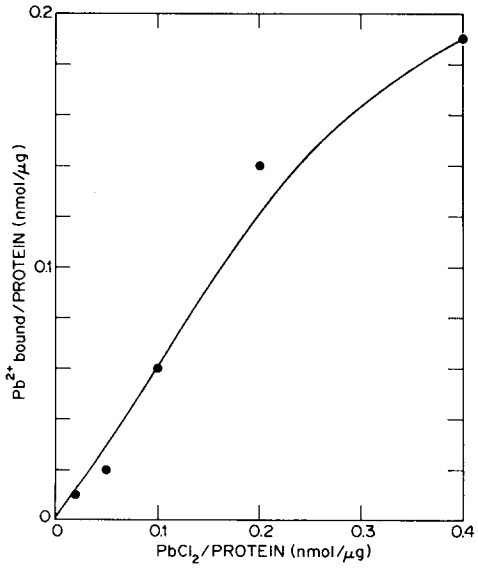

Fig. 4. ^{210}Pb binding to erythrocyte membranes as a function of total lead added per μg membrane protein. The conditions are described in Materials and Methods. A 40 min exposure of membranes to $PbCl_2$ preceded washing. The data are obtained after two washings of the membranes and represent the mean of duplicate experiments that differ by less than 20%.

membrane fragments. This may account for the slow development of inhibition and failure of 40 000-fold dilution of the lead to reverse the inhibition of the ($Na^+ + K^+$)-ATPase. The ability of chelators to partially reverse the inhibition may be related to the large stability constants of lead-chelator complexes (e.g., approx. $1 \cdot 10^{18}$ for Pb^{2+}-EDTA complexes [16]). Vesicle formation may also account for the reduced ability of chelators in restoring activity in contrast to preventing inhibition. If, indeed, vesicles do form, some may be inside-out while others are right-side-out [17]. Lead is thought to act near the Na^+ site on phosphorylatable enzyme units oriented toward the cytoplasmic side of the membrane [18]. The degree of reversibility would be proportional to the percentage of inside-out vesicles (where chelators would have easy access to inhibitory lead) and to the permeability of the membranes to the chelators. Further experiments are necessary to examine these possibilities.

It is of interest to examine the constraints in seeking a possible relationship between these in vitro results and human lead intoxication, since alteration of cation transport could result in increased red cell fragility. The present data show that the extent of inhibition of ($Na^+ + K^+$)-ATPase can be related to the ratio of lead to membrane protein. Significant inhibition (10%) is observed at a ratio of 20 pmol Pb^{2+}: μg protein (Fig. 2). In patients with clear clinical evidence of lead intoxication, the whole blood levels of lead range from 100 to 1000 μg/100 g whole blood or higher [19], more than 90% of which is sequestered with the red cells [8,20]. If one assumes 320 mg membrane protein per 100 g whole blood (yield data in Materials and Methods) and that all of the lead is accessible to the ($Na^+ + K^+$)-ATPase (which, of course, may not be so since much of the lead may be bound to proteins such as hemoglobin [21]), then in clinical lead intoxication at most 1.5—15 pmol Pb^{2+}: μg membrane protein would be available for inhibiton. Although it is difficult to compare in vitro conditions with those found in vivo, in cases of extreme toxicity there may be a significant inhibition of the ($Na^+ + K^+$)-ATPase, whereas at the lower limit of toxicity no clear evidence for inhibition exists based on these data. We have shown, however, that lead inhibition of the ($Na^+ + K^+$)-ATPase develops slowly. It is possible that chronic exposure to lower levels of lead in vivo might result in a higher degree of inhibition than that which is observed after the 40 min in vitro incubation. Instability of the enzyme complicates such studies in vitro. Indeed, Angle and McIntire [8] report that the erythrocyte ($Na^+ + K^+$)-ATPase of urban children is decreased when red cell lead levels exceed 40 μg/100 ml (greater than 0.7 pmol Pb^{2+}: μg membrane protein). These findings are supported by Hasan et al. [7]. In both reports the decrease was slight and without a linear correlation between the low blood lead levels and enzyme activity. Further studies are necessary to assess the relationship between higher blood levels and degree of inhibition of the ($Na^+ + K^+$)-ATPase.

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References

1 Goldberg, A. (1972) Br. J. Haematol. 23, 521—524
2 Buchet, J.P., Roels, M., Hubermont, G. and Lauwerys, R. (1976) Toxicology 6, 21—34
3 Aub, J.C., Reznikoff, P. and Smith, D.E. (1924) J. Exp. Med. 40, 151—172
4 Vincent, P.C. (1958) Aust. J. Exp. Biol. 36, 589—602
5 Riordan, J.R. and Passow, H. (1971) Biochim. Biophys. Acta 249, 601—605
6 Selhi, H.S. and White, J.M. (1975) Postgrad. Med. J. 51, 765—769
7 Hasan, J., Vihko, V. and Hernberg, S. (1967) Arch. Environ. Health 14, 313—318
8 Angle, C.R. and McIntire, M.S. (1974) Environ. Health Perspect. 7, 133—137
9 Siegel, G.J., Fogt, S.K. and Hurley, M.J. (1977) in Membrane Toxicity (Miller, M.W. and Shamoo, A.E., eds.), pp. 465—493, Plenum Press, New York
10 Siegel, G.J. and Fogt, S.K. (1977) J. Biol. Chem. 252, 5201—5205
11 Nechay, B.R. and Saunders, J.P. (1978) J. Toxicol. Environ. Health 4, 147—159
12 Post, R.L., Merrit, C.R., Kinsolving, C.R. and Albright, C.D. (1960) J. Biol. Chem 235, 1796—1802
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Siegel, G.J. and Albers, R.W. (1967) J. Biol. Chem. 242, 4972—4979
15 Segel, I.M. (1975) Enzyme Kinetics, p. 127, John Wiley, New York
16 Couer, A., Bouvet, R. and Boucherle, A. (1962) Lyon Pharm. 13, 287—305
17 Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255—257
18 Siegel, G.J., Fogt, S.K. and Iyengar, S. (1978) J. Biol. Chem. 253, 7207—7211
19 Chisolm, J.J., Jr. (1968) J. Pediatr. 73, 1—38
20 Ryder, H.W., Cholak, J. and Kehoe, R.A. (1947) Science 106, 63—64
21 Barltrop, D. and Smith, A. (1971) Experientia 27, 92—93