Studies on the interactions of human pancreatic elastase 2 with human α_1 -proteinase inhibitor and α_1 -antichymotrypsin

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The interactions of human pancreatic elastase 2 with α_1 -proteinase inhibitor and α_1 -antichymotrypsin were compared by studies *in vitro*. The equimolar complexes obtained between the enzyme and either inhibitor were relatively stable at 25 °C since they could be visualized for up to 5 days by an electrophoretic method. However, in both cases, a slow dissociation occurred with release of active enzyme. As the k_{ass} rate constants are of the same order of magnitude, with a slightly lower value for α_1 -proteinase inhibitor when compared with α_1 -antichymotrypsin [$(5.6 \pm 1.2) \times 10^5$ and $(8.9 \pm 1.3) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively], partition of human pancreatic elastase 2 between both inhibitors in human plasma is mainly dependent on their respective concentrations. A comparative study by crossed immunoelectrophoresis of the interactions of this enzyme with the two inhibitors contained in normal human plasma and in a mimetic mixture of pure inhibitors was carried out. This allowed the visualization of complexes with either inhibitor. Formation of such a complex with α_1 -antichymotrypsin had never been demonstrated previously. The patterns obtained are similar when working with normal plasma or with the synthetic mixture, suggesting that, in the conditions used, α_1 -proteinase inhibitor and α_1 -antichymotrypsin are the main inhibitors of human pancreatic elastase 2 in the plasma sample. However, it is also shown that part of the enzyme may be taken up by α_2 -macroglobulin, which is responsible for the remaining enzyme activity on a synthetic substrate. The present work suggests that, according to the delay times of inhibition of human pancreatic elastase 2 calculated from the normal plasma concentrations of α_1 -proteinase inhibitor and α_1 -antichymotrypsin, a significant role can be assigned to both inhibitors. Moreover, the role of α_1 -antichymotrypsin would be enhanced in α_1 -proteinase-inhibitor deficiency.

INTRODUCTION

Human pancreatic elastase 2 (HPE 2; EC 3.4.21.36) is a chymotrypsin-like serine proteinase which is the major elastolytic enzyme found in the pancreas [1]. Its zymogen form, referred to as proelastase 2, has been shown to occur in normal human plasma as a complex with α_1 -proteinase inhibitor (α_1 -PI), which has been detected with radioimmunoassay [2,3]. An extensive study of the formation of this complex has been performed *in vitro*, including isolation and characterization with the use of ^{125}I -proelastase 2 [4]. Interactions between HPE 2 and plasma proteinase inhibitors such as α_2 -macroglobulin and α_1 -PI have also been investigated elsewhere [5]. Another study showed that the concentration of immunoreactive HPE 2 was increased in human plasma from acute pancreatitis when compared with normal plasma and that the enzyme was found complexed with α_1 -PI [6]. α_1 -PI is the major serine proteinase inhibitor found in human plasma [7]; its interactions with numerous human and animal proteinases, including chymotrypsin-like enzymes such as human and bovine chymotrypsins and leukocyte cathepsin G, have been extensively investigated [8,9]. The molar combining ratio for this inhibitor and HPE 2 has been determined [5,10] but no kinetic data have been published concerning this interaction.

We previously reported by studies *in vitro* [11] that

another plasma serine proteinase inhibitor, α_1 -antichymotrypsin (α_1 -Achy), was able to inactivate porcine pancreatic elastase 1 and HPE 2, which both have the capacity to digest fibrous elastin completely. However, the complete inhibition of porcine pancreas elastase needed a markedly higher inhibitor : enzyme molar ratio (5.5:1) when compared with the ratio required to inactivate HPE 2 (1.25:1) [11]. This finding led us to suggest that α_1 -Achy, which is an acute phase plasma protein [12], could contribute to the regulatory balance between proteinase inhibitors and HPE 2 during the inflammatory processes associated with pancreatic diseases. The primary role of α_1 -Achy in humans has been shown to be the specific regulation of chymotrypsin-like proteinases, including leukocyte cathepsin G and mast cell chymase [13]. Interactions between this inhibitor and human cathepsin G and bovine pancreatic chymotrypsin have also been described by us [14,15]. In our previous work, the formation of a complex between α_1 -Achy and HPE 2 was demonstrated [11].

The purpose of the present work was to study more precisely the interactions of α_1 -PI and α_1 -Achy with HPE 2. The stoichiometries of inhibition as well as the association rate constants were compared. The stability of the complexes formed was checked in a time-course study. Simultaneous complex formation with both inhibitors was shown by crossed immunoelectrophoresis after the addition of HPE 2 to normal human plasma.

MATERIALS AND METHODS

HPE 2 (a generous gift from Dr. M. Rabaud, Unité 8 de l'INSERM, Pessac, France) was prepared by the procedure described in [1]; its activity was estimated as in [11]. Bovine pancreatic trypsin (Sigma; EC 3.4.21.4) was active-site titrated using *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride (Sigma) according to [16]. Human α_1 -Achy was purified by a procedure previously described [14]. Human α_1 -PI was also prepared using immunoaffinity chromatography [17]. The two inhibitors were kept frozen in 0.01 M-sodium phosphate buffer (pH 7.4) containing 0.3 M-NaCl and 0.02% NaN₃ until use. Their concentrations were determined according to [18] with a correction for the carbohydrate part of the molecules: 24% for α_1 -Achy and 13% for α_1 -PI.

Incubations of inhibitor-enzyme mixtures and enzyme assays were performed at 25 °C. All experiments were done in duplicate. α_1 -PI was first assayed with bovine trypsin: trypsin (0.13 μ M) was reacted for 5 min with varying amounts of inhibitor in 0.2 M-Tris/HCl buffer, pH 8.0, and the residual enzyme activity was measured with benzoyl-arginine *p*-nitroanilide (0.5 mM). The molar combining ratio of α_1 -PI and HPE 2 was determined as previously described for α_1 -Achy [11] (enzyme concentration 105 nM). To calculate the inhibitor : enzyme molar ratios throughout the experiments, we took into account the total content of both inhibitors but only the amount of active enzyme. Determination of the rate constant for the association of HPE 2 with α_1 -PI was performed as in [11] with the following modifications: the enzyme concentration was 10.5 nM and the more sensitive substrate glutaryl-Ala-Ala-Pro-Leu *p*-nitroanilide (Bachem) [19] was chosen (concentration 1 mM in the assays).

Processing of the inhibitor-enzyme mixtures and analytical polyacrylamide-gel electrophoreses were carried out as previously described [11]. M_r values were estimated to be 59000, 54000 and 25000 for α_1 -Achy, α_1 -PI and HPE 2 respectively. Crossed immunoelectrophoreses were performed as in [15]. Specific antisera against α_1 -Achy and α_1 -PI were from Dako. The precipitation peaks obtained were sufficiently distinguishable; thus it was possible to perform simultaneous analysis by mixing both specific antisera in the second dimension.

RESULTS

Reaction of human α_1 -PI with HPE 2

Stoichiometry of the inhibition. Inhibitory activity of α_1 -PI was first checked on bovine trypsin which had been previously titrated for active enzyme content. The inhibitor : enzyme molar ratio was found to be 1.16:1 at the equivalence point. For the reaction between α_1 -PI and HPE 2, a 10 min incubation time was determined to be sufficient to obtain a linear inhibition curve up to 4% residual enzyme activity. The extrapolation of the linear part of the curve (not shown) led to zero enzyme activity at an inhibitor : enzyme molar ratio equal to 1.28:1, which was slightly higher than the ratio given above for bovine trypsin.

Association rate constant. A time-dependent inhibition of HPE 2 by α_1 -PI was obtained by working with an enzyme concentration 10 times lower than for the

determination of the stoichiometry of the reaction. In the experimental conditions used, a $k_{\text{ass.}}$ value of 5.6 ± 1.2 (S.D.) $\times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated (two independent determinations, each with ten experimental points).

Comparative studies between α_1 -PI and α_1 -Achy for their reaction with HPE 2

Recovery of enzyme activity from mixtures. Mixtures of α_1 -PI and α_1 -Achy with HPE 2 were prepared in inhibitor : enzyme molar ratios chosen to have a slight excess (10%) of inhibitor over enzyme. Aliquots were analysed for dissociation of the complexes by measuring enzyme activity released at various periods. The activity of a control enzyme solution prepared and incubated in the same conditions as the inhibitor-enzyme mixtures greatly decreased with time and only 36% of the initial activity at time zero remained at the end of the experiment. Therefore, enzyme activity recovered after dissociation from the complexes was expressed by comparison with the initial activity of the control solution. Starting from a zero enzyme activity in both mixtures at time zero, 19% and 17% were measured in the α_1 -PI-HPE 2 mixture after 3 and 7 days of incubation respectively, whereas 11% and 12% activity were released in the α_1 -Achy-HPE 2 mixture at the same times.

Time-course study of the dissociation of the complexes by electrophoretic analyses. First, the stability of the complexes of either inhibitor with enzyme was investigated by electrophoretic analysis. Then, each inhibitor was studied for its ability to bind the enzyme dissociated from the complex formed with the other inhibitor. Thus, mixtures of α_1 -PI with HPE 2 were added to α_1 -Achy and vice versa. A time-course study was performed for both inhibitor-enzyme reactions as follows: mixtures of either inhibitor and enzyme prepared as above were started off at different times; at the end of the incubation times, each mixture was divided into two and processed as described in the legend to Fig. 1.

In Fig. 1(a) are shown the results of the time-course study of mixtures of α_1 -PI and HPE 2. It is noticeable that the equimolar complex of M_r approx. 73000 is relatively stable, since it remains visible over 5 days although the intensity of its band decreases after 3 days. The slight excess of active inhibitor is present up to 4 h of incubation and has disappeared after 24 h. A degraded complex of M_r approx. 60000 is distinctly seen up to 24 h of incubation. In samples 1-6, the band of M_r approx. 50000 is attributed to modified inactive α_1 -PI: its intensity increases with time of incubation and its migration becomes slightly faster when there is no active inhibitor left in the mixture. As no enzyme activity was detected spectrophotometrically up to 4 h of incubation, the band of M_r 25000 in samples 1-3 is only due to the inactive part of the enzyme. The intensity of this band increases with time: this shows that some enzyme is released from the complex and seen as a free form when the excess of inhibitor has been used up (samples 4-6).

In Fig. 1(b) are shown the results obtained when α_1 -Achy was added at the end of the same incubation times as in Fig. 1(a). Addition of α_1 -Achy does not increase the dissociation rate of the complex, which also persists up to 5 days. However free enzyme released from the α_1 -PI-HPE 2 complex after more than 24 h (previ-

Fig. 1. Time-course study of the reaction of HPE 2 with α_1 -PI by SDS/polyacrylamide-gel electrophoresis on a 5–30% gradient gel

The mixtures were prepared with a 10% excess of inhibitor and kept at 25 °C for the various incubation times indicated further. In (a), samples 1–6 were immediately added to phenylmethanesulphonyl fluoride and analysed, whereas in (b) samples 1'–6' were added to α_1 -Achy in equimolar amount as α_1 -PI, incubated for an additional 10 min, then added to phenylmethanesulphonyl fluoride and analysed. Incubation times: 1 and 1', 10 min; 2 and 2', 1 h; 3 and 3', 4 h; 4 and 4', 24 h; 5 and 5', 3 days; 6 and 6', 5 days. Lane M, M_r markers [phosphorylase b (94000), bovine albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α -lactalbumin (14400)]. Only this sample was reduced. Lane C, control mixture (α_1 -PI + inactivated HPE 2); lane E, HPE 2; lanes I, α_1 -PI; lane I', α_1 -Achy; lane m', mixture of α_1 -Achy and HPE 2 incubated for 10 min. The minor band of M_r approx. 14000 is as in [11].

ously observed in Fig. 1a, samples 4–6) is taken up by α_1 -Achy and appears as a complex with this inhibitor (M_r approx. 77000), easily seen in samples 5' and 6'. The same kind of results (not shown) were obtained when the time-course study was performed on mixtures of α_1 -Achy and HPE 2. The complex was relatively stable; release of enzyme was only noticed after 24 h of incubation. After addition of α_1 -PI to the mixtures, the complex formation between this latter inhibitor and the active enzyme released from the former complex occurred to a lesser extent in this experiment.

Simultaneous reaction of HPE 2 with the two inhibitors

The reaction of HPE 2 with α_1 -PI and α_1 -Achy was followed by crossed immunoelectrophoresis with antisera specific for either inhibitor. We chose, first, a natural mixture of these two inhibitors, human plasma, in which α_1 -PI and α_1 -Achy concentrations were determined by

electroimmunoassay. We studied the complex formation after the addition to normal human plasma of two different amounts of HPE 2 (left side of Fig. 2a: panels b and c). Moreover, we compared these patterns with those obtained (right side of the Fig. 2a: panels e and f) when a mimetic mixture of pure α_1 -PI and α_1 -Achy, in the same concentrations as in the normal plasma, was added to the same amounts of HPE 2. The antisera amounts were chosen to obtain easily visible precipitation peaks for both components. Consequently, the relative peak heights do not account for the respective concentrations of the two inhibitors. The original samples without enzyme added (Fig. 2a, panels a and d) give similar patterns, apart from the small IgA- α_1 -PI complex present in normal plasma (panel a) as previously described [20]. The amount of enzyme added to the mixtures analysed in panels (b) and (e) led to inhibitor: enzyme molar ratios equal to 3.6:1 and 0.7:1 for α_1 -PI

and α_1 -Achy respectively; there was thus an excess of total inhibitor content. On the contrary, in panels (c) and (f) the aforementioned molar ratios were 0.91:1 and 0.18:1 for α_1 -PI and α_1 -Achy respectively. Addition of HPE 2 to the normal plasma or to the mimetic mixture produced the simultaneous formation of a complex with each inhibitor, visualized as a precipitation peak having in both cases a slower electrophoretic mobility than the native inhibitor. The heights of these peaks increase with the amount of enzyme. The non-complexed peaks visualized after the addition of enzyme are composed of two species in panels (b) and (e), i.e. active inhibitor in excess and modified inactive inhibitor obtained after the reaction; they are only composed of modified inactive inhibitor in panels (c) and (f) since there is no active inhibitor left in these mixtures. When comparing the free/complexed form peak height ratios for both inhibitors, the results are similar in panels (c) and (f). On the contrary this ratio, at least for α_1 -PI, markedly differs in panels (b) and (e) (free/complexed = 4 and 3 respectively) suggesting that, in these conditions, the synthetic mixture cannot exactly mimic the inhibitors reacting with the enzyme in normal plasma.

The titration curve of HPE 2 by human plasma is shown in Fig. 2(b). The patterns shown in Fig. 2(a) for panels (b) and (c) are related on the inhibition curve to points b and c respectively; these two points refer to 18% and 16% residual activity of the enzyme respectively. It is noteworthy that from point c, which corresponds to the bottom of the linear inhibition curve obtained with small plasma volumes, the activity reaches a minimum and further rises with increasing amounts of plasma. This has been attributed to the formation of a complex of the enzyme with α_2 -macroglobulin [5]; this complex retains enzyme activity for small synthetic substrates.

DISCUSSION

The inhibitor : enzyme combining ratio for α_1 -PI and HPE 2, determined to be 1.28:1, compares well with the value of 1.25:1 obtained for α_1 -Achy reacting with the same enzyme [11]. For easier comparison of both inhibitors, the molar ratio calculations refer to the total inhibitor concentrations since α_1 -Achy cannot be calibrated with a titrated enzyme [15]. However, if the α_1 -PI concentration is corrected assuming a 1:1 stoichiometry with titrated trypsin, the molar combining ratio of α_1 -PI with HPE 2 becomes 1.1:1. This slightly higher ratio could be explained by a slight dissociation of the complex with the latter enzyme, as shown in the present paper. Moreover, the above value is somewhat higher than the 1:1 stoichiometry found in earlier reports [5,10]: this could be attributed to the different experimental

conditions used. In fact, these slight discrepancies might be considered to be within the experimental errors.

The present work deals with the native, functional species of α_1 -PI. Previous studies have brought evidence that oxidation of this inhibitor on the methionine residue of its active site led to partial and total inactivation of the inhibitor towards human leukocyte and porcine pancreatic elastases respectively [9]. There is an open question as to whether oxidized α_1 -PI is still able to inactivate HPE 2.

When the two inhibitors are compared for their reaction with HPE 2, the determined k_{ass} values are of the same order of magnitude, with a higher value for α_1 -Achy ($8.9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) than for α_1 -PI ($5.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). Therefore, α_1 -Achy can act as a protecting agent against the elastolytic activity of HPE 2. From the k_{ass} value determined for α_1 -PI in this study, and assuming a concentration of $50 \mu\text{M}$ in normal plasma, a delay time of inhibition *in vivo* was calculated to be 0.18 s, according to [21]. For a concentration of α_1 -Achy in normal plasma of $9 \mu\text{M}$, the calculation with the k_{ass} value determined in our previous work [11] led to a delay time *in vivo* of 0.62 s, which is short enough to assign to this inhibitor a physiological function [21]. Consequently, due to its higher concentration in normal plasma, α_1 -PI is the most efficient inhibitor of HPE 2, when both inhibitors are compared. On the other hand, in the case of α_1 -PI genetic deficiency (ZZ subjects), where the plasma concentration is 7 times lower than in normal plasma, α_1 -Achy could play a significant role as a substitute for the inactivation of HPE 2.

The complexes formed between α_1 -PI or α_1 -Achy and HPE 2 are relatively stable and only a small release of enzyme activity was quantified after 7 days of incubation from mixtures prepared in a slight excess of inhibitor. A time-course study performed by electrophoretic analyses allowed the visualization of the dissociation products from the complexes formed with either inhibitor. When a second inhibitor was added to the first inhibitor-enzyme mixture, another complex was formed and was visualized after the longest incubation times studied. It corresponded to the binding of the active enzyme dissociated from the first complex. This was observed whatever the order of inhibitor addition. It was also noticed that dissociation did not occur more rapidly in the presence of the second inhibitor. This is consistent with the above results which emphasize the stability of either complex. Thus a common mechanism of interaction is involved when either inhibitor associates with HPE 2.

Interactions between HPE 2 and plasma proteinase inhibitors have been investigated previously [5,6]. However none of these authors sought the existence of any complex with α_1 -Achy in plasma. Several lines of

Fig. 2. Reaction of HPE 2 with proteinase inhibitors in normal plasma and in a synthetic mixture (a) and titration of HPE 2 by human plasma (b)

(a) Reaction of HPE 2 in normal human plasma (left side) and in a mimetic concentration mixture of pure α_1 -PI and α_1 -Achy (right side) studied by crossed immunoelectrophoresis. Reaction mixtures were incubated for 10 min at 25 °C. Aliquots were analysed using a mixture of the antisera against α_1 -PI and α_1 -Achy (1.25 and $0.31 \mu\text{l} \cdot \text{cm}^{-2}$ respectively). The amounts applied were as follows: α_1 -PI, $5.2 \mu\text{g}$; α_1 -Achy, $1.1 \mu\text{g}$; enzyme, none in a and d, $0.66 \mu\text{g}$ in b and e, $2.65 \mu\text{g}$ in c and f. (b) Titration of the enzyme by human plasma. Increasing amounts of plasma were reacted with $20 \mu\text{l}$ of enzyme solution ($5.3 \mu\text{g}$) in 0.2 M -Tris/HCl buffer, pH 8.0, (1 ml final volume) for 10 min at 25 °C and the residual enzyme activity was measured after the addition of the substrate succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (1 mM). Points b and c on the inhibition curve correspond to the same inhibitor : enzyme molar ratios as in the reaction mixtures analysed in panels b and c respectively of part (a).

evidence are presented in the present paper which provide strong support for the assertion that, in addition to α_1 -PI, α_1 -Achy is also able to give rise to complex(es) with HPE 2 in human plasma. Our comparative study of the enzyme reaction in human plasma and in a synthetic mixture of both inhibitors by crossed immunoelectrophoresis would suggest that, in the conditions of enzyme concentration we used, α_2 -macroglobulin does not play a major role for enzyme inhibition. As shown on the inhibition curve of Fig. 2(b), this inhibitor is however able to bind a significant part of HPE 2. This might explain the differences observed between the patterns of panels (b) and (e) in Fig. 2(a). Therefore the respective roles of the three inhibitors would depend on the enzyme concentration in plasma.

Complexes of α_1 -Achy have been formed in human serum *in vitro* with bovine chymotrypsin [22] and leukocyte cathepsin G [23]; in our study a complexed form of this inhibitor with HPE 2 has been demonstrated. The reports of Largman *et al.* [2] indicated that proelastase 2, which was synthesized in and secreted from the pancreas, reached the blood where it was able to complex with α_1 -PI. It would be of interest to investigate whether α_1 -Achy is also capable of binding that zymogen in blood.

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REFERENCES

1. Largman, C., Brodrick, J. W. & Geokas, M. C. (1976) *Biochemistry* **15**, 2491–2500
2. Largman, C., Brodrick, J. W., Geokas, M. C., Johnson, J. H. & Fassett, M. (1980) *Am. J. Physiol.* **238**, G177–G182
3. Geokas, M. C., Brodrick, J. W., Johnson, J. H. & Largman, C. (1977) *J. Biol. Chem.* **252**, 61–67
4. Largman, C., Brodrick, J. W., Geokas, M. C., Sischo, W. M. & Johnson, J. H. (1979) *J. Biol. Chem.* **254**, 8516–8523
5. Gustavsson, E. L., Ohlsson, K. & Olsson, A. S. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 169–176
6. Borgstrom, A., Kukora, J. & Ohlsson, K. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 633–640
7. Heimburger, N., Haupt, H. & Schwick, H. G. (1971) in *Proceedings of the International Research Conference on Proteinase Inhibitors* (Fritz, H. & Tschesche, H., eds.), pp. 1–21, Walter de Gruyter, New York
8. Aubry, M. & Bieth, J. (1977) *Clin. Chim. Acta* **78**, 371–380
9. Beatty, K., Bieth, J. & Travis, J. (1980) *J. Biol. Chem.* **255**, 3931–3934
10. Tyndall, M., Largman, C., Brodrick, J. W. & Geokas, M. C. (1977) *Biochem. Biophys. Res. Commun.* **74**, 857–861
11. Laine, A., Davril, M., Rabaud, M., Vercaigne-Marko, D. & Hayem, A. (1985) *Eur. J. Biochem.* **151**, 327–331
12. Aronsen, K. F., Ekelund, G., Kindmark, C. O. & Laurell, C. B. (1972) *Scand. J. Clin. Lab. Invest.* **29** (Suppl. 124), 127–136
13. Travis, J., Bowen, J. & Baugh, R. (1978) *Biochemistry* **17**, 5651–5656
14. Laine, A., Hayem, A. & Davril, M. (1984) in *Marker Proteins in Inflammation* (Arnaud, P., Bienvenu, J. & Laurent, P., eds.), vol. 2, pp. 171–179, Walter de Gruyter, Berlin and New York
15. Laine, A., Davril, M. & Hayem, A. (1984) *Eur. J. Biochem.* **140**, 105–111
16. Chase, T. & Shaw, E. (1970) *Methods Enzymol.* **19**, 20–27
17. Vercaigne-Marko, D., Carrère, J., Ducourouble, M. P., Davril, M., Laine, A., Amouric, M., Figarella, C. & Hayem, A. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 37–45
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
19. Del Mar, E. G., Largman, C., Brodrick, J. W., Fassett, M. & Geokas, M. C. (1980) *Biochemistry* **19**, 468–472
20. Laurell, C. B. & Thulin, E. (1975) *Scand. J. Immunol. Suppl.* **2**, 7–12
21. Bieth, J. G. (1980) *Clin. Respir. Physiol.* **16** (Suppl.), 183–195
22. Ohlsson, K. (1971) *Scand. J. Clin. Lab. Invest.* **28**, 5–11
23. Ohlsson, K. & Akesson, U. (1976) *Clin. Chim. Acta* **73**, 285–291