Biochem. J. (1982) **203**, 149–153
Printed in Great Britain

The kinetics of hydrolysis of some extended *N*-aminoacyl-L-arginine methyl esters by human plasma kallikrein

Evidence for subsites S_2 and S_3

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(*Received 7 October 1981/Accepted 16 December 1981*)

Subsites in the S_2–S_4 region were identified in human plasma kallikrein. Kinetic constants ($k_{cat.}$,K_m) were determined for a series of seven extended *N*-aminoacyl-L-arginine methyl esters based on the *C*-terminal sequence of bradykinin (-Pro-Phe-Arg) or $(Gly)_n$-Arg. The rate-limiting step for the enzyme-catalysed reaction was found to be deacylation of the enzyme. It was possible to infer that hydrogen-bonded interactions occur between substrate and the S_2–S_4 region of kallikrein. Insertion of L-phenylalanine at residue P_2 demonstrates that there is also a hydrophobic interaction with subsite S_2, which stabilizes the enzyme–substrate complex. The strong interaction demonstrated between L-proline at residue P_3 and subsite S_3 is of greatest importance in the selectivity of human plasma kallikrein. The purification of kallikrein from Cohn fraction IV of human plasma is described making use of endogenous Factor XIIf to activate the prekallikrein. Kallikreins I (M_r 91000) and II (M_r 85000) were purified 170- and 110-fold respectively. Kallikrein I was used for the kinetic work.

One of the functions of human plasma kallikrein (EC 3.4.21.8) is the excision of the nonapeptide vasodilator bradykinin from high-molecular-weight kininogen. In its action on this substrate the enzyme acts with a high degree of specificity, cleaving only three peptide bonds (Kerbiriou & Griffin, 1979; Nakayasu & Nagasawa, 1979). Kallikreins have a 'trypsin-like' primary specificity for L-arginine peptide bonds and to a smaller extent L-lysine peptide bonds (Fiedler & Leysath, 1979). In view of its high degree of selectivity in its action on high-molecular-weight kininogen, it seems likely that additional or 'secondary' specificity is conferred by a sequence of subsites on the enzyme adjacent to the primary specificity site (S_1).

The sequence of high-molecular-weight kininogen in the vicinity of the *C*-terminal sequence of the excised bradykinin moiety is -Pro-Phe-Arg-Ser-Val-, the Arg–Ser bond being susceptible to cleavage. The object of the present study is a kinetic investigation of the enzyme subsites designated S_2 and S_3 (Schechter & Berger, 1967), which are believed to interact with phenylalanine (P_2) and proline (P_3) residues of the substrate. The synthetic L-arginine methyl ester substrates Ac-Arg-OMe,HCl, Ac-Phe-Arg-OMe,HCl and Ac-Pro-Phe-Arg-OMe,HCl were used to investigate the overall interactions at subsites S_2 and S_3. The existence of any hydrogen-bonded interactions at these subsites was investigated by using the 'stripped' substrates Ac-Gly-Arg-OMe,HCl and Ac-Gly-Gly-Arg-OMe,HCl. The 'selectively stripped' substrates Ac-Gly-Phe-Arg-OMe,HCl and Ac-Pro-Gly-Arg-OMe,HCl were used both to investigate specific interactions of phenylalanine with subsite S_2 and of proline with subsite S_3 respectively, and also to investigate the possibility of any subsite–subsite interactions occurring with the full substrate Ac-Pro-Phe-Arg-OMe,HCl. The *N*-acetyl blocking group was used for reasons given previously (Green & Tomalin, 1976).

The purification of human plasma kallikrein has been reported by a number of authors (Sampaio *et al.*, 1974; Heber *et al.*, 1978; Nagase & Barrett, 1981). In the present work a preparation method utilizing Cohn fraction IV of human plasma (Cohn *et al.*, 1946) as the raw material was used. Fraction IV comprises both fraction IV-1, which contains prekallikrein (Sampaio *et al.*, 1974), and fraction IV-4, which contains kallikrein (Heber *et al.*, 1978) and Factor XIIf or prekallikrein activator (Kuwahara, 1980). It is expected that activation of prekallikrein should be possible in a suspension of Cohn fraction IV by adsorbing Factor XIIf on glass Ballotini beads (Davies *et al.*, 1967). The activated

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prekallikrein is then adsorbed on (soya-bean trypsin inhibitor)–Sepharose. The free enzyme is regenerated by treatment with 1 M-benzamidine, followed by dialysis, and further purified by gel chromatography.

Two active forms of the enzyme, namely kallikrein I (M_r 88000) and kallikrein II (M_r 85000), have been reported (Mandle & Kaplan, 1977; Nagase & Barrett, 1981). Kallikrein I was used for the kinetic work.

Materials and methods

Materials

Cohn fraction IV obtained by cold ethanol precipitation of human plasma (Kistler & Nitschmann, 1962) was obtained from Blood Products Laboratory, Elstree, Herts., U.K. Sepharose 4B was from Pharmacia, Hounslow, Middlesex, U.K. Ultrogel AcA-44 was from LKB Instruments, South Croydon, Surrey, U.K. Trypsin inhibitor (soya-bean) type I-S, 5,5'-dithiobis-(2-nitrobenzoic acid), 4-methylumbelliferone and Brilliant Blue G were from Sigma Chemical Co., Poole, Dorset, U.K. Benzamidine hydrochloride hydrate and CNBr were from Aldrich Chemical Co., Gillingham, Dorset, U.K. 4-Methylumbelliferyl *p*-guanidinobenzoate was synthesized by Hill (1979). N^{α}-Benzyloxycarbonyl-L-lysine thiobenzyl ester hydrochloride was synthesized from L-lysine (Green & Shaw, 1979; Hill, 1979). Ac-Arg-OMe,HCl, Ac-Phe-Arg-OMe,HCl, Ac-Pro-Phe-Arg-OMe,HCl, Ac-Gly-Arg-OMe,HCl, Ac-Gly-Gly-Arg-OMe,HCl, Ac-Gly-Phe-Arg-OMe,HCl and Ac-Pro-Gly-Arg-OMe,HCl were synthesized from their constituent amino acids. These substrates were synthesized by the mixed-anhydride coupling of N^{α}-t-butoxycarbonyl-protected amino acids to N^{G}-nitroarginine methyl ester by using isobutyl chloroformate as coupling reagent as described by Hill (1979). Selective cleavage of the t-butoxycarbonyl group was by treatment with hydrogen chloride in ethyl acetate (Katsoyannis & Schwartz, 1977). N^{α}-Acetylation of the peptides was performed by using *N*-acetoxysuccinimide, and catalytic hydrogenation was used to deprotect the guanido function of L-arginine in the final products as described by Hill (1979). The products from the intermediate steps in the syntheses were found to be homogeneous by t.l.c., gave sharp melting points and satisfactory elemental analyses (C, H, N). The ester substrates were also found to be susceptible to rapid ester hydrolysis by bovine trypsin (EC 3.4.21.4) at pH 8.00, the extent of the rapid hydrolysis being equivalent, within experimental error, to that calculated from weighing. The extent of racemization of the L-arginine moiety in each substrate was thus negligible. The optical rotation of each substrate, $[\alpha]_D$, was found to be constant, within experimental error, for all separate preparations.

Glassware used in the enzyme purification was silicone-treated with Repelcote from Hopkin and Williams, Chadwell Heath, Essex, U.K., unless otherwise stated.

Preparation of (soya-bean trypsin inhibitor)–Sepharose 4B

Sepharose 4B (100ml) was activated with CNBr (March *et al.*, 1974) and coupled to soya-bean trypsin inhibitor (250mg) overnight at 4°C in 0.1 M-$NaHCO_3$, pH 8.3, containing 0.5 M-NaCl. The gel was washed, and shaken with 1 M-ethanolamine, pH 9.0, for 2h at room temperature, The gel was then washed with coupling buffer and three cycles each of: (i) 0.1 M-sodium acetate buffer, pH 4.0, containing 1 M-NaCl; (ii) 0.1 M-sodium borate buffer, pH 8.0, containing 1 M-NaCl. (Soya-bean trypsin inhibitor)–Sepharose 4B was stored in 10mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl and 0.02% (w/v) NaN_3.

Enzyme assays

Kallikrein activity was routinely assayed spectrophotometrically with N^{α}-benzyloxycarbonyl L-lysine thiobenzyl ester hydrochloride as substrate (Green & Shaw, 1979). Enzymic hydrolysis was monitored at 412nm in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, with the use of 5,5'-dithiobis-(2-nitrobenzoic acid) as chromogen for the benzylmercaptan, in a Perkin–Elmer model 402 spectrophotometer. One unit of activity hydrolysed 1 μmol of substrate/min under the conditions described.

Active-site titration

The operational molarity of the stock enzyme solutions was determined by spectrofluorimetric titration with 4-methylumbelliferyl *p*-guanidinobenzoate in a Perkin–Elmer–Hitachi model 204 spectrofluorimeter by the method of Jameson *et al.* (1973).

Protein determination

Protein concentrations were determined either by using Coomassie Blue G-250 by the method of Sedmak & Grossberg (1977), with crystalline bovine serum albumin as standard, or by absorption measurement at 280nm. $A^{1\%}_{1cm,280}$ for purified plasma kallikrein was taken to be 10.6 (Nagase & Barrett, 1981).

Purification of plasma kallikrein

All purification procedures were conducted at 4°C unless otherwise stated.

In order to activate the prekallikrein, fraction IV (1000g) was dissolved in 40mM-sodium phosphate buffer, pH 7.4, containing 0.6 M-NaCl (1.5 litres),

and the solution was stirred with 0.1mm glass Ballotini beads (not silicone-treated; 50g) for 90min. After removal of the Ballotini beads by filtration, (soya-bean trypsin inhibitor)–Sepharose 4B (20g) was added and the mixture was stirred for 5min. The affinity adsorbent was removed by filtration and washed with 10mM-sodium phosphate buffer, pH7.4, containing 1M-NaCl and 2μM-benzamidine (20 litres), followed by 10mM-sodium phosphate buffer, pH7.4, containing 0.15M-NaCl (10 litres), until the absorbance of the eluent at 280nm was less than 0.02. The enzyme was regenerated from the gel by washing with 10mM-sodium phosphate buffer, pH7.4, containing 0.15M-NaCl and 1M-benzamidine, followed by removal of the benzamidine by exhaustive dialysis against distilled water. The resulting solution was concentrated to 10ml by ultrafiltration (Stirred Cell, PM10 membrane; Amicon Corp., Lexington, MA, U.S.A.). In order to remove high-molecular-weight kallikreins (Colman *et al.*, 1969), a sample (1ml) was applied to an Ultrogel AcA-44 gel column (95cm × 1.6cm) previously equilibrated at room temperature with 10mM-sodium phosphate buffer, pH6.0, containing 0.1M-NaCl, and the kallikrein was eluted with the same buffer. Active forms in the molecular-weight region 80000–95000 were collected and dialysed against 1mM-sodium phosphate buffer, pH6.0, containing 0.1M-NaCl, and stored frozen at −20°C until further use. The results of the purification are summarized in Table 1.

Kinetic measurements

The progress of ester hydrolysis was followed on a Radiometer pH-stat comprising a pH-meter (PHM 62), titration unit (TTT 60c), automatic burette (ABU 13), titration assembly (TTA 60) and recorder (REC 61/REA 160). The jacketed reaction vessel was maintained at 37 ± 0.1°C by circulation from a Grant LE8 thermostatically controlled water bath. All measurements were performed under an atmosphere of CO_2-free N_2 saturated with water. The pH electrode was calibrated against two standard buffers (Perrin & Dempsey, 1974). Alkali solutions were prepared by diluting a measured volume of carbonate-free 25M-NaOH solution into previously boiled-out distilled water (Vogel, 1961). The absolute molarities of titrant solutions were determined by titration against potassium hydrogen phthalate solution (Vogel, 1961). Stock substrate solutions and subsequent dilutions were made with 0.1M-NaCl to give a final volume of 10ml. Adjustment to pH8.00 was made with 0.15M-NaOH. Kallikrein I solution (100μl) was added, and the uptake of alkali at pH8.00 was recorded as a function of time. After correction for any non-enzymic hydrolysis, initial velocities were calculated and values of $k_{cat.}$ and K_m were computed by the method of Wilkinson (1961). Product-partitioning studies were conducted by using Ac-Pro-Phe-Arg-OMe,HCl in the presence of 2M-methanol (Seydoux & Yon, 1967, 1971) in a similar manner to that described above.

Results and discussion

Purification of human plasma kallikreins I and II

It was found that kallikreins may be purified from Cohn fraction IV of human plasma, after activation of prekallikrein with glass-adsorbed endogenous Factor XIIf. This method is believed to be preferential and closer to physiological activation than activation procedures involving the use of acetone (Nagase & Barrett, 1981) and acid (Sampaio *et al.*, 1974).

The results for the purification procedure are summarized in Table 1. It was found that kallikreins

Table 1. *Purification of human plasma kallikreins I and II*
See the Materials and methods section for experimental details.

Step	Esterase activity (munits/ml)*	Protein concentration (mg/ml)	Specific activity (munits/mg)	Purification factor
Cohn fraction IV	27.9	13.5†	2.07	—
Glass-activated fraction IV	27.9	13.5†	2.07	—
(Soya-bean trypsin inhibitor)–Sepharose 4B	338	2.16†	157	75.6
Ultrogel AcA-44 step				
(i) Kallikrein I	22.5	0.064‡	352	170
(ii) Kallikrein II	10.5	0.046‡	228	110
(iii) High-molecular-weight kallikreins§	7.5	0.012‡	625	302

* N^{α}-Benzyloxycarbonyl-L-lysine thiobenzyl ester hydrochloride in 0.1M-Tris/HCl buffer, pH8.0, containing 0.1M-NaCl (Green & Shaw, 1979).
† Coomassie Blue G-250 (Sedmak & Grossberg, 1977).
‡ A_{280}.
§ Colman *et al.* (1969).

I and II (Mandle & Kaplan, 1977) could be resolved, and were found to give single bands on sodium dodecyl sulphate / polyacrylamide - gel electrophoresis, giving M_r values of 91000 ± 2000 and 85000 ± 2000 respectively. These values are in agreement with those previously reported (Mandle & Kaplan, 1977; Nagase & Barrett, 1981).

Active-site titration

The concentration of active enzyme was readily determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973). The pre-steady-state burst was rapid, being complete within the time of mixing. Enzyme concentrations could be determined with an accuracy of $\pm 4\%$

Enzyme kinetics

The mechanism of action of serine proteinases is known to involve acylation and deacylation of an active-site serine residue in accordance with the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_{+2}} ES' \xrightarrow{k_{+3}} E + P_2$$

Here ES' is the acyl-enzyme, in this case an *N*-aminoacyl-L-arginyl-kallikrein. In order to determine whether the rate-limiting step is acylation or deacylation, for hydrolysis of methyl esters, product-partitioning studies were conducted in the presence and in the absence of methanol. This nucleophile competes with water in the deacylation process. If deacylation is the rate-limiting step (i.e. $k_{+2} \gg k_{+3}$), it can be shown that the observed $k_{cat.}$ value for the formation of free acid (P_2) will be independent of nucleophile concentration, whereas the observed value of K_m will increase linearly with nucleophile concentration (Seydoux & Yon, 1967). Table 2 gives the results of such a study with Ac-Pro-Phe-Arg-OMe,HCl as substrate and 2 M-methanol as nucleophile. Since $k_{cat.}$ was found to be unaltered within the limits of experimental error, it is concluded that deacylation is rate-limiting for human plasma kallikrein I. It seems reasonable to draw the same conclusion for the other substrates with this enzyme. It is therefore possible to relate the observed values of $k_{cat.}$ and K_m to the rate constants in the above scheme as follows:

$$k_{cat.} = k_{+3} \quad (1)$$

$$K_m = [(k_{-1} + k_{+2})/k_{+1}](k_{+3}/k_{+2}) \quad (2)$$

Kinetic results for all seven substrates are given in Table 3. It can be seen that the $k_{cat.}$ and K_m values for the extended substrates differ markedly from those for Ac-Arg-OMe,HCl, indicating that the P_2 and P_3 residues are involved in subsite interactions with the enzyme. The values of $k_{cat.}$ for the extended substrates remain fairly constant within the range 34–$42\,s^{-1}$, indicating that the rate of deacylation is remarkably insensitive to the substitution of the amino acid residues at P_2 and P_3. It is clear that the enhanced catalytic efficiency for the extended substrates as reflected in $k_{cat.}/K_m$ values is a result of higher overall binding of the substrates produced by subsite interactions. Eqn. (2) shows that the steady-state constant $[(k_{-1} + k_{+2})/k_{+1}]$ is greater than the observed Michaelis constant (K_m) by a factor of k_{+2}/k_{+3}. Despite the fact that the measured values of k_{+3} are fairly constant, it is still not possible to conclude whether the measured K_m values predominantly reflect changes in the enzyme–substrate binding affinity (k_{-1}/k_{+1}), the rate of enzyme acylation (k_{+2}) or a combination of both effects.

Extension of the basic Ac-Arg-OMe,HCl substrate to Ac-Gly-Arg-OMe,HCl causes a decrease in the observed K_m value from 1.22 mM to 0.56 mM. It is not possible to conclude whether this is a result of the decreased value of k_{+3} for the extended substrate or a true increase in substrate binding strength. In either case it is clear that the effect is caused by hydrogen-bonded interactions of the Ac-Gly moiety with the enzyme in the S_2/S_3 subsite region. Extension of Ac-Gly-Arg-OMe,HCl to Ac-Gly-Gly-Arg-OMe,HCl has no effect on the deacylation rate, but decreases K_m from 0.56 mM to 0.18 mM. Increased binding strength is thus caused by further hydrogen bonds to the enzyme surface in the S_3/S_4 region. Similar hydrogen bonds to the enzyme in the S_2–S_4 region have been inferred for both β-trypsin (EC 3.4.21.4) and chymotrypsin A_α (EC 3.4.21.1) (Green & Tomalin, 1976; Hill & Tomalin,

Table 2. *Kinetic constants for the partitioning studies with Ac-Pro-Phe-Arg-OMe,HCl in the presence of 2 M-methanol by kallikrein I at 37°C at pH 8.00 in 0.1 M-NaCl*

Concn. of methanol (M)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)	No. of runs
0	42.2 ± 0.7	0.014 ± 0.001	2900	22
2	40.9 ± 0.8	0.035 ± 0.002	1180	21

Table 3. *Kinetic constants for the hydrolysis of* L-*arginine methyl ester substrates catalysed by human plasma kallikrein I at 37°C at pH 8.00 in 0.1* M-*NaCl*

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)	No. of runs
Ac-Arg-OMe,HCl	81 ± 18	1.22 ± 0.34	66	20
Ac-Gly-Arg-OMe,HCl	40.0 ± 1.2	0.560 ± 0.025	72	20
Ac-Gly-Gly-Arg-OMe,HCl	40.0 ± 0.7	0.180 ± 0.007	221	21
Ac-Phe-Arg-OMe,HCl	33.9 ± 1.4	0.160 ± 0.014	207	21
Ac-Gly-Phe-Arg-OMe,HCl	34.1 ± 0.6	0.150 ± 0.006	222	21
Ac-Pro-Gly-Arg-OMe,HCl	39.4 ± 0.4	0.027 ± 0.001	1460	21
Ac-Pro-Phe-Arg-OMe,HCl	42.4 ± 0.7	0.014 ± 0.001	2900	22

1981), but these interactions were mainly responsible for enhancement of the rate of deacylation.

Insertion of the L-phenylalanine residue at P_2 in Ac-Phe-Arg-OMe,HCl and Ac-Gly-Phe-Arg-OMe,HCl results in a significant decrease in the rate of deacylation of the corresponding 'stripped' substrates from $40\,s^{-1}$ to $34\,s^{-1}$ and a corresponding decrease in the K_m values to 0.16 and 0.15 mM respectively. It is clear that there is a hydrophobic binding site at P_2 that binds the L-phenylalanine residue, in addition to the possibility of hydrogen-bonded interactions. The former interaction significantly stabilizes the enzyme–substrate complex relative to the transition state for deacylation of the enzyme, since the deacylation rate is significantly lowered.

Insertion of the L-proline residue at P_3 in Ac-Pro-Gly-Arg-OMe,HCl, on the other hand, does not have a significant effect on the deacylation rate, but is associated with the largest decrease in the K_m value from 0.18 mM for Ac-Gly-Gly-Arg-OMe,HCl to 0.027 mM. The L-proline interaction at residue P_3 has a much larger effect on the selectivity of human plasma kallikrein than the interaction of L-phenylalanine at residue P_2. Combination of these two effects in Ac-Pro-Phe-Arg-OMe,HCl is noteworthy, since the concerted interaction gives rise to a very low value of K_m (0.01 mM) and a significantly enhanced deacylation rate ($42\,s^{-1}$). It is possible that the locked configuration of the Ac-Pro moiety when bound to the enzyme at subsites S_3/S_4 causes the L-phenylalanine residue bound at subsite S_2 to adopt the most favourable conformation for transition-state stabilization rather than enzyme–substrate-complex stabilization.

We thank the Medical Research Council for a research studentship (to P. R. L.) and a grant for equipment (to G. T.), Blood Products Laboratory for plasma fractions and Mr. Ian Geller for technical assistance.

References

Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946) *J. Am. Chem. Soc.* **68**, 459–475

Colman, R. W., Mattler, L. & Sherry, S. (1969) *J. Clin. Invest.* **48**, 11–22

Davies, G. E., Holman, G. & Lowe, J. S. (1967) *Br. J. Pharmacol. Chemother.* **29**, 55–62

Fiedler, F. & Leysath, G. (1979) in *Kinins II* (Fujii, S., Moriya, H. & Suzuki, T., eds.), pp. 261–271, Plenum Press, London and New York

Green, G. D. J. & Shaw, E. (1979) *Anal. Biochem.* **93**, 223–226

Green, G. D. J. & Tomalin, G. (1976) *Eur. J. Biochem.* **68**, 131–137

Heber, H., Geiger, R. & Heimburger, N. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 659–669

Hill, C. R. (1979) Ph.D. Thesis, Victoria University of Manchester, pp. 302–326

Hill, C. R. & Tomalin, G. (1981) *Biochim. Biophys. Acta* **660**, 65–72

Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A. & Elmore, D. T. (1973) *Biochem. J.* **131**, 107–117

Katsoyannis, P. G. & Schwartz, G. P. (1977) *Methods Enzymol.* **47E**, 501–578

Kerbiriou, D. M. & Griffin, J. H. (1979) *J. Biol. Chem.* **254**, 12020–12027

Kistler, P. & Nitschmann, H. S. (1962) *Vox Sang.* **7**, 414–424

Kuwahara, S. S. (1980) *Transfusion* **20**, 433–439

Mandle, R. & Kaplan, A. P. (1977) *J. Biol. Chem.* **252**, 6097–6104

March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152

Nagase, H. & Barrett, A. J. (1981) *Biochem. J.* **193**, 187–192

Nakayasu, T. & Nagasawa, S. (1979) *J. Biochem. (Tokyo)* **85**, 249–258

Perrin, D. D. & Dempsey, B. (1974) *Buffers for pH and Metal Ion Control*, 1st edn., p. 39, Chapman and Hall, London

Sampaio, C., Wong, S.-C. & Shaw, E. (1974) *Arch. Biochem. Biophys.* **165**, 133–139

Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162

Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552

Seydoux, F. & Yon, J. (1967) *Eur. J. Biochem.* **3**, 42–56

Seydoux, F. & Yon, J. (1971) *Biochem. Biophys. Res. Commun.* **44**, 745–751

Vogel, A. I. (1961) *A Textbook of Quantitative Inorganic Analysis*, 3rd edn., pp. 241–243, Longmans, London

Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332