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THE EFFECT OF ZINC *IN VIVO* AND *IN VITRO* ON THE ACTIVITIES OF ANGIOTENSIN CONVERTING ENZYME AND KININASE-I IN THE PLASMA OF RATS

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Abstract—Two groups of rats were pair fed, for 18 days, diets containing either 2.6 (Zn deficient) or 100 mg Zn/kg (control diet). Plasma was assayed spectrophotometrically for the activity of kininase-I and angiotensin converting enzyme (ACE) in the presence of varying concentrations of added Zn^{2+}. Zinc deficient rats had only 76% of the activity of both kininase-I and ACE compared with zinc supplemented control rats. There was a significant linear relationship between enzyme activity and concentration of zinc in plasma for both enzymes. When zinc was added to the enzyme incubation mixture for zinc deficient rats, the activity of ACE increased by 73% and that of kininase-I by 33%. This Zn^{2+}-stimulated increase in enzyme activity was negatively correlated with the *in vivo* concentration of zinc in plasma, and a plateau in enzyme activity was seen at concentrations of plasma zinc that were commensurate with normal zinc status (over 14 μmole/l). The results demonstrate that the activities of both kininase-I and ACE are dependent on the concentration of zinc *in vivo* and *in vitro*, and suggest that information concerning the concentration of zinc in plasma and assay solutions be a prerequisite for the use of these enzymes in the clinical diagnosis of disease states. The results also showed that the activity of ACE and kininase-I in plasma could be used for the biochemical diagnosis of a suboptimal zinc status.

The activity of angiotensin converting enzyme (ACE, EC 3.4.15.1) in plasma is used for the clinical diagnosis and monitoring of certain diseases, notably sarcoidosis [1–3] and hypertension [4, 5]. Kininase-I activity has not been monitored in such cases, but both ACE and kininase-I are involved in the regulation of vasoactive hormones. Human plasma ACE is a zinc-containing carboxy-terminal peptidyl dipeptidase of mol. weight 140,000 [6] that inactivates bradykinin and cleaves the dipeptide His-Leu from the c-terminal end of angiotensin-I to form angiotensin-II [6, 7]. Kininase-I (also known as carboxypeptidase N, arginine carboxypeptidase, serum carboxypeptidase B and anaphylatoxin inactivator) [8, 9] exists in the serum of several species as a tetrameric complex of 280,000 mol. weight [8]. It removes the C-terminal arginine of bradykinin to form vaso-inactive fragments. Kininase-I has been shown to contain zinc [10], and is activated by Co and Ni ions and inhibited by high concentrations of Zn, Cd and Hg *in vitro* [11].

We have recently shown that both ACE and kininase-I activities are decreased in plasma of zinc deficient rats [12]. This has been verified for ACE by Reeves and O'Dell [13], and these findings have raised questions concerning the clinical use of ACE as an indicator of disease states.

The aim of the following study was to further describe the relationship between the *in vivo* and *in vitro* concentration of zinc and the peptidase activity of ACE and kininase-I in plasma. Zinc concentration *in vivo* was altered by offering diets that were either deficient or adequate in zinc, while *in vitro* it was altered by adding Zn^{2+} to the enzyme incubation mixture.

MATERIALS AND METHODS

Enzyme assays. ACE activity was measured in plasma using a modification of the method of Cushman and Cheung [14, 15]. Briefly, 100 μl of sample was incubated for 60 min at 37° with 50 μl 0.5 M potassium phosphate buffer (pH 8.3) containing 0.75 M NaCl, and 100 μl of 8.4 mM substrate solution (Hippuryl-L-His-L-Leu, Serva Feinbiochemika). The reaction was stopped with 200 μl 1 M HCl and the mixture extracted with 1.5 ml ethyl acetate. One ml of the solvent was removed, dried at 120° and the hippuric acid remaining was redissolved in 3 ml 1 M NaCl solution. The solution was placed in quartz cuvettes and the concentration of hippuric acid determined using u.v. absorbance at 228 nm in a Zeiss spectrophotometer (deuterium lamp). Blanks received 200 μl of 1 M HCl before substrate addition. Enzyme activity was calculated as follows:

$$EU = \frac{(ODs - ODb)}{60} \times \frac{1}{9.8 \times 10^{-3}} \times \frac{1}{0.67} \times \frac{1}{0.91} \times \frac{3}{0.10}$$

= nmoles hippuric acid formed/min/ml plasma

where: ODs is the sample absorbance after 60 min; ODb is the blank absorbance after 60 min; 9.8×10^{-3}

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is the molar extinction coefficient (litre/mol/cm); 0.67 is the fractional volume of ethyl acetate removed; 0.91 is the efficiency of hippuric acid extraction; 0.1 is the sample volume (ml); 3 is the final water volume (ml).

Kininase-I peptidase activity was assayed using a modification of the method of Skidgel and Erdos [16] with 25 mM hippuryl-L-Lys as substrate (Sigma, Munich). The main modification was that a pre-incubation with cobalt was not carried out because it was considered that cobalt would mask the effects of zinc. Otherwise the conditions were as described by Skidgel and Erdos [16]. The method is essentially similar to that for ACE except that 0.25 M Tris buffer (pH 7.5) is used in place of phosphate buffer, and no NaCl is added to the incubation mixture or the final water solution. Enzyme activity is calculated as for ACE, with change in absorbance at 228 nm being measured over 1 hr. Enzyme units are nmoles hippuric acid formed per min. per ml of plasma at 37°. For both ACE and kininase-I, internal standards of bulked rat plasma and human serum were included at the beginning and end of each assay to serve as within- and between-assay controls.

For the *in vitro* studies, a stock solution of 100 ppm Zn^{2+} (1.54 mmole/l) was prepared by dissolving 22 mg $ZnSO_4.7H_2O$ in 50 ml distilled water. Varying amounts of this solution were added to the incubation mixture immediately prior to substrate addition and the added volume adjusted to a maximum of 50 μl by adding distilled water.

The concentration of zinc in the diet was determined by atomic absorption spectroscopy (AAS) after dry ashing in a muffle furnace [17]. Zinc in the incubation mixture and in diluted plasma (1:5 with water) was determined by direct aspiration using flame AAS.

Enzyme specificity was assessed using the inhibitors MK422 (Merck Sharp & Dohme) and 1,10 *o*-phenanthroline (Sigma, Munich). These inhibitors were prepared at working strength solutions of 3×10^{-4} and 1 mmole/l, respectively. A 25 μl aliquot of inhibitor solution was added to the incubation mixture immediately prior to the addition of substrate.

Zinc deficiency studies. Forty-two Sprague Dawley rats of both sexes and weighing 131 ± 4 g were divided into two groups containing equal proportions of males and females. They were fed for 18 days on a casein based (EDTA-extracted casein) diet containing either 2.6 (Zn deficient diet) or 100 mg Zn/kg (control diet) as added $ZnSO_4$. The composition and method of preparation of the diet have been described elsewhere [18]. Rats were caged in groups of 3 or 4 and control rats were pair fed to zinc deficient rats on a daily basis. This consisted of feeding control rats the same amount of diet as previously eaten by their deficient "pair" on the preceding day. Cages were plastic with stainless steel grid lids. Distilled drinking water containing 0.014% NaCl was provided ad libitum throughout the experiment.

Whole blood was collected by heart puncture from anaesthetized rats (10 mg Inactin i.p. per 100 g body weight) at slaughter. Blood was drawn into syringes containing 160 I.U. heparin (Karl Roth, Karlsruhe) and immediately centrifuged at 1200 *g* for 5 min. The plasma was stored frozen at −80°.

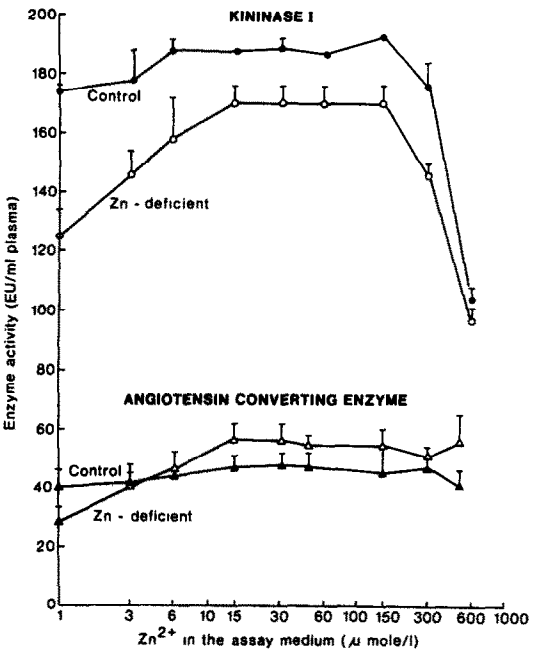

Fig. 1. The activity of kininase-I (o) and ACE (△) at different *in vitro* concentrations of Zn^{2+} ($\bar{X}$ + S.E.M.). Closed symbols represent control rats (15.7 μmole Zn/l plasma) and open symbols zinc-deficient rats (5.7 μmole Zn/l plasma).

Results were analysed by regression analysis or by analysis of variance [19].

RESULTS

A. *Effect of* Zn^{2+} *on* in vitro *enzyme activity*

No zinc could be detected in the buffer or substrate solution. Addition of Zn^{2+} to the incubation mixture of both ACE and kininase-I at concentrations ranging from 3 to 600 μmole Zn/l, resulted in activity dose–response curves rising to a plateau at approximately 15 μmole/l and showing inhibitory effects for kininase-I after 150 μmole/l (Fig. 1). Zinc deficient rats had initial enzyme activities that were 25–30% lower than control rats but showed a greater relative response to Zn^{2+} than control rats. For ACE this resulted in a higher final activity in plasma from deficient rats. For kininase-I, the activity in plasma from deficient rats after Zn^{2+} addition *in vitro* was approximately 90% ($P < 0.001$) of control values.

B. *Effect of dietary zinc on enzyme activity*

Despite pair feeding, zinc deficient rats had growth rates of 2.0 ± 0.2 g/day while control rats gained 2.8 ± 0.1 g/day ($P < 0.01$). Deficient rats also showed skin lesions symptomatic of zinc deficiency. The concentration of zinc in plasma did not differ between the sexes and fell from an initial value of 15.7 μmole/l down to 5.7 μmole/l by the end of the experiment (Table 1). This fall was associated with a 24% decline in enzyme activity for both ACE and kininase-I (Table 1). For zinc deficient rats, the *in*

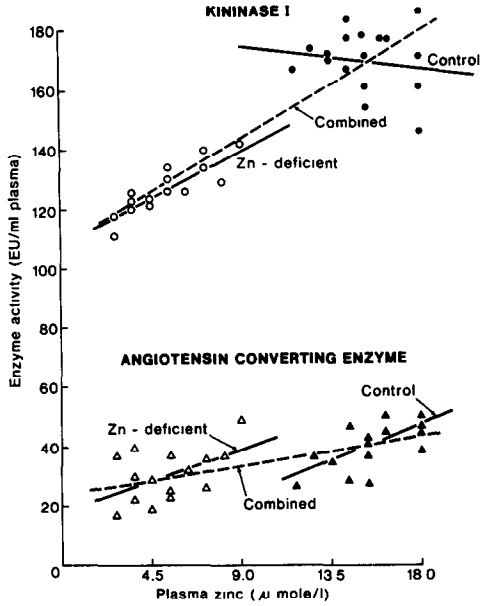

Fig. 2. The activity of kininase-I (o) and ACE (△) at different *in vivo* concentrations of zinc in plasma. Symbols are as for Fig. 1. The following equations are based on Y (enzyme activity; EU/ml plasma) vs X (plasma zinc; μmole/l). Probability values are < 0.001, < 0.01 and < 0.05 for ***, ** and *, respectively.

Treatment group	Regression equation	N	r^2	P
Kininase-I				
Zn deficient	Y = 109 + 3.4X	15	0.69	***
control	Y = 182 − 0.7X	17	0.02	NS
combined	Y = 109 + 3.8X	32	0.79	***
ACE				
Zn deficient	Y = 19 + 2.1X	15	0.24	NS
control	Y = 2 + 2.4X	15	0.40	*
combined	Y = 24 + 1.0X	30	0.41	***

vitro addition of either 30 or 15 μmoles Zn/l, respectively, to the enzyme incubation mixture of ACE and kininase-I resulted in an activity increase of 73% for ACE and 33% for kininase-I (Table 1). For control rats, the respective increases after Zn^{2+} were only

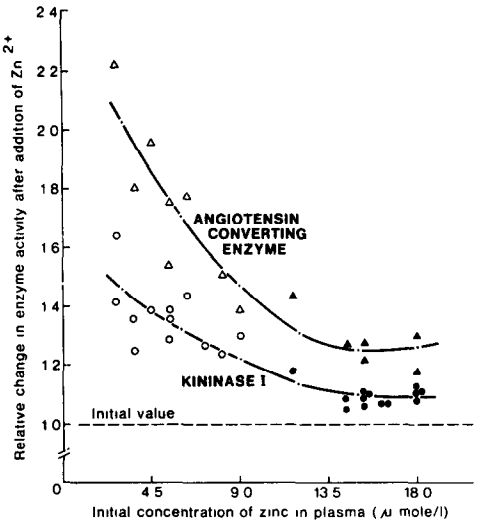

Fig. 3. Relative increase in the activity of kininase-I (o) and ACE (△) in plasma after Zn^{2+} addition *in vitro* (30 and 15 μmoles/l, respectively). Symbols are as for Fig. 1. Regression equations are based on Y (enzyme activity; EU/ml plasma) versus X (plasma zinc; μmole/l). A quadratic equation gave the best fit for both enzymes. Activity for kininase-I was described by: $Y = 1.62 - 0.06X + 1.6 \times 10^{-3} X^2$; $r^2 = 0.82$, (***) while for ACE, $Y = 2.45 - 0.15X + 4.5 \times 10^{-3} X^2$; $r^2 = 0.87$ (***).

30 and 9%. For ACE, this difference in response resulted in zinc deficient rats tending to have higher enzyme activities than control rats after *in vitro* Zn^{2+} addition (P = 0.09). For kininase-I, enzyme activity was higher in control rats than deficient rats both before and after Zn^{2+} addition *in vitro*.

There was a significant linear relationship between the concentration of zinc in plasma and enzyme activity for both ACE and kininase-I (Fig. 2), with kininase-I having a larger correlation coefficient than ACE for this regression (r^2 of 0.79, cf. 0.41), with the respective standard errors for slope representing 10 and 20% of the mean (not shown). For deficient rats, there was a significant linear relationship between enzyme activity and the concentration of zinc in plasma only for kininase-I, whereas for control rats, only ACE showed a significant correlation

Table 1. Effects of zinc deficiency on plasma zinc concentration and on the activities of ACE and kininase-I in the presence and absence of added *in vitro* zinc ($\bar{X} \pm$ S.E.M.)

	Zn^{2+} added to assay medium (μmole/l)	Dietary Zn (mg/kg) 2.6	100	P
Plasma Zn (μmoles/l)	—	5.7 ± 0.5	15.7 ± 0.5	***
ACE (EU)	0	31.1 ± 2.1	40.8 ± 2.0	***
	30	53.9 ± 3.2	44.7 ± 3.8	NS
Ratio 30/0		1.73 ± 0.1	1.30 ± 0.1	**†
Kininase-I (EU)	0	127.4 ± 2.3	170.0 ± 2.3	***
	15	168.0 ± 3.3	185.1 ± 2.7	**
Ratio 15/0		1.33 ± 0.1	1.09 ± 0.1	***

The ratio 30/0 and 15/0 represents the relative increase in activity after the addition of Zn^{2+} *in vitro*.

*** P < .001; ** P < .01; * P < .05; NS, P > .05

† Zn^{2+} was added to the plasma from 6 control rats. Thus, the ratio of 1.30 represents the relative increase in ACE activity seen only in those 6 rats.

coefficient. When enzyme activity was expressed as a ratio of activity after zinc addition to activity before zinc addition, both enzymes showed a highly significant negative correlation with plasma zinc concentration. The increase in enzyme activity in response to the addition of Zn^{2+} *in vitro* was greater for ACE than kininase-I (Fig. 3), with both enzymes showing no further increase in activity at *in vivo* plasma zinc concentrations greater than approximately 14 μmole/l (0.9 mg/l).

C. *Effect of inhibitors on enzyme activity*

The addition of the ACE inhibitor MK422 caused 90% inhibition of ACE at inhibitor concentrations greater than 3×10^{-3} μmole/l, while at concentrations up to 0.118 μmole/l, it had no effect on kininase-I activity. At 1.18 μmole/l MK422, the activity of kininase-I in plasma from control rats (initial activity of 130 EU) was reduced by 24%. This compared with 42% inhibition for a sample of human plasma, also with an initial kininase-I activity of 130 U/l. Phenanthroline did not inhibit kininase-I until concentrations in the incubation mixture exceeded 40 μmole/l. At 400 μmole/l, activity in plasma from control rats and in human serum was decreased by 82 and 52%, respectively. The inhibition of ACE activity by phenanthroline was not tested.

DISCUSSION

The results demonstrate that the activities of both ACE and kininase-I in plasma are dependent on the zinc status of the animal and on the concentration of zinc in the enzyme incubation mixture. These findings are in agreement with those of Reeves and O'Dell [13]. The dependence of enzyme activity on zinc concentration makes the enzymes potentially useful for the biochemical diagnosis of zinc deficiency, and supports earlier suggestions that some of the pathological lesions of zinc deficiency may be related to a decreased activity of ACE and kininase-I in plasma [12]. The results also have implications concerning a possible role for zinc in the regulation of blood pressure, although it is unclear as to whether the observed decreases in enzyme activity would have any effect on blood pressure through reduced angiotenin II concentration since renin is the rate limiting enzyme of this system. Of immediate relevance, however, is that no published method for either enzyme recommends the addition of zinc to the assay medium, although cobalt is recommended for kininase-I [16]. Activity of ACE in serum and plasma is used in clinical studies as an indicator of disease states such as sarcoidosis [1–3], silicosis [20], and in anti-hypertensive drug treatment programmes [4, 21–25]. A change in the *in vitro* enzyme activity is generally interpreted as indicating a change in the *in vivo* enzyme concentration. However, hypozincaemia appears to be a relatively common phenomena, being associated with pregnancy [26], oral contraceptives, alcoholic cirrhosis, active tuberculosis, Downs Syndrome, uremia [27], infection, myocardial infarction [28] and neoplastic diseases [29]. Hypozincaemia could be contributing to variations in plasma ACE activity in conflicting ways. For example, evidence from this experiment indicates that plasma ACE activity will be higher or lower in hypozincaemia depending on the presence or absence of zinc contamination in the assay medium, with optimal enzyme activity at *in vitro* zinc concentrations of between 15 and 150 μmole/l. Since plasma will contribute only about 1.5 (Zn deficient rats) or 3.0 μmole Zn/l (control rats) if used at 1:5 dilution, most enzyme assays for ACE and kininase-I will give suboptimal values unless zinc contamination is present in the buffer or substrate solutions. This is obviously an unsatisfactory situation for clinical or experimental studies. In most species, the normal concentration of zinc in plasma is between 15 and 30 μmole/l, a range shown in the current experiment to be coincident with optimal ACE and kininase-I activity *in vitro*. In this context the results indicate that the plasma activities of ACE and kininase-I could be useful for the biochemical diagnosis of zinc deficiency. Kininase-I activity appears to be a better predictor than ACE of the concentration of zinc in plasma from deficient rats, perhaps because ACE is more sensitive to *in vitro* zinc contamination. However, this responsiveness of ACE to the presence of zinc resulted in the potential for a different kind of diagnosis for zinc status. This method is based on the high correlation coefficient for the negative relationship between plasma zinc and the relative increase in activity of ACE after *in vitro* Zn^{2+} addition. Roth and Kirchgessner [30] proposed a similar diagnostic approach using plasma alkaline phosphatase, except that their method involved activity measurements before and 3 days after zinc injection. In contrast to alkaline phosphatase, results from the current experiment suggest that synthesis (or release into plasma) of ACE is not zinc dependent, and in fact raise the possibility of increased *de novo* synthesis of plasma apoenzyme in zinc deficient rats. For this reason, the Zn^{2+}-stimulated increase in ACE activity is probably a more sensitive indicator of suboptimal zinc status than an absolute activity measurement of ACE or kininase-I. However, the usefulness of any such approach depends upon the constancy of the relationship between the concentration of zinc in plasma and enzyme activity, and this needs to be determined for situations in addition to the dietary manipulations used in this experiment. Of interest is whether the short term changes in plasma zinc concentration, seen in various disease states, for example, are a physiological mechanism related to the regulation of plasma peptidase activity.

In addition to stimulating enzyme activity, zinc inhibits both ACE and kininase-I activity when the metal is present at high concentrations. Decreased ACE activity was observed at Zn^{2+} concentrations above 500 μmole/l, a result similar to that of Cushman and Cheung using rabbit lung ACE [14]. Inhibition of ACE activity has been reported at Zn^{2+} levels of 20 μmole/l, but only when the assay procedure required a 40-fold dilution of the serum [13]. Similarly, kininase-I was inhibited at concentrations of Zn^{2+} greater than 150 μmole/l in the present experiment, but this was an order of magnitude greater than that observed by Erdos *et al.* [11] using 1:40 diluted serum containing 100 μmoles Co/l. Thus the results suggest that both ACE and kininase-I are

relatively insensitive to inhibition by Zn^{2+} provided the enzyme is not too diluted. At high dilutions (i.e. 1:40), added zinc appears to interfere at relatively low concentrations (under 20 μmole/l).

Results relating to the inhibition of the enzymes by the ACE inhibitor MK422, and by the metal chelator *o*-phenanthroline, agree with those published elsewhere. For ACE, others have shown that MK422 inhibits activity at 1 nmole/l; a concentration that is six orders of magnitude less than that for inhibition by phenanthroline [31–33]. For kininase-I, Erdos *et al.* [11] and Skidgel *et al.* [34], obtained a 50% inhibition of activity in human serum with 1 mmole/l *o*-phenanthroline, a result similar to that seen with 0.4 mmole/l in the current experiment. (It should be pointed out that phenanthroline is not a specific inhibitor for kininase-I, and DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (MGTA) would have been preferable.) MK422 has not previously been tested against kininase-I, although another ACE inhibitor, Captopril, at 10 μmole/l has been shown not to affect human plasma kininase-I activity [34]. In the current experiment, MK422 at 1.2 μmole/l inhibited kininase-I by 35% in rat plasma and by 42% in human serum. Thus, ACE inhibitors will inhibit kininase-I *in vitro*, albeit at higher concentrations (1000-fold) than for ACE. The significance of this for subjects receiving oral MK422 remains to be determined.

In conclusion, the dependence of ACE and kininase-I activities on zinc status suggest that these enzymes may prove useful for the biochemical diagnosis of zinc deficiency. In addition, the results have implications for the use of ACE as an indicator of disease states, and suggest that the concentration of zinc in plasma be determined in all cases where activities of ACE or kininase-I are to be used for clinical diagnosis. Where relevant, zinc should be added to the assay media, at a concentration calculated to give optimal activity for the particular assay procedure used.

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REFERENCES

1. J. Lieberman, *Am. J. Med.* **59**, 365 (1975).
2. J. Lieberman, A. Nosal, L. A. Schlessner and A. Sastre-Foken, *Am. Rev. Respir. Dis.* **120**, 329 (1979).
3. F. K. Rømer, *Act. med. Scand. Suppl.* **690**, (1983).
4. H. H. Rotmensch, P. H. Vlasses and R. K. Ferguson, *Israel J. med. Sci.* **18**, 981 (1982).
5. H. Schweisfurth, A. Kment, H. Dahlheim and B. E. Strauer, *Klm. Wochenschr.* **60**, 49 (1982).
6. J. J. Lanzillo and B. L. Fanburg, *Biochemistry* **16**, 5491 (1977).
7. R. L. Soffer and E. H. Sonnenblick, *Progr. cardiovasc. Dis.* **21**, 167 (1978).
8. G. Oshima, J. Kato and E. G. Erdös, *Biochim. biophys. Acta* **365**, 344 (1974).
9. E. G. Erdös, in *Handbook of Experimental Pharmacology, XXV Supplement* (Ed. E. G. Erdös), p. 427. Springer, Heidelberg (1979).
10. T. H. Plummer and M. Y. Hurwitz, *J. biol. Chem.* **253**, 3907 (1978).
11. E. G. Erdös, E. M. Sloane and I. M. Wohler, *Biochem. Pharmac.* **13**, 893 (1964).
12. C. L. White, J. Pschorr, I. C. M. Jacob, N. von Lutterotti and H. Dahlheim, in *Trace Element Metabolism in Man and Animals-5.* (Eds C. F. Mills, I. Bremner and J. K. Chesters), p. 65. Commonwealth Agricultural Bureau, Slough, U.K. (1985).
13. P. G. Reeves and B. L. O'Dell, *Clin. Chem.* **31**, 581 (1985).
14. D. W. Cushman and H. S. Cheung, *Biochem. Pharmac.* **20**, 1637 (1971).
15. J. H. Rosenthal, B. Pfeifle, M. L. Michailov, J. Pschorr, I. C. M. Jacob and H. Dahlheim, *Hypertension* **6**, 383 (1984).
16. R. A. Skidgel and E. G. Erdös, in *Methods of Enzymatic Analysis,* Vol. V, (Ed. H. U. Bergmeyer), p. 60. Verlag Chemie Weinheim (1984).
17. H. P. Roth and M. Kirchgessner, *Res. Exp. Med. (Berl.)* **174**, 283 (1979).
18. J. Pallouf and M. Kirchgessner, *Z. Teirphysiol. Tierernährg. Futtermittelkde.* **28**, 128 (1971).
19. G. W. Snedecor and W. G. Cochran, in *Statistical Methods*, 6 Edn. Iowa State University Press, Ames, IA (1967).
20. H. Nordman, H. Koskinen and B. Fröseth, *Chest* **86**, 203 (1984).
21. D. Klaus, R. Lederle and B. Braun, in *Pharmacology and Clinical Use of Angiotensin I Converting Enzyme Inhibitors* (Eds. F. Gross, and R. K. Liedtke) p. 92. Gustav Fischer, Stuttgart (1980).
22. M. A. Petty, J. L. Reid and S. H. K. Miller, *Life Sci.* **26**, 2045 (1980).
23. H. Gavras, J. Biollaz, B. Waeber, H. R. Brunner, I. Gavras, H. Sackel, F. Charocopos and R. O. Davies, *Clin. Sci.* **61**, 281S (1981).
24. S. G. Chrysant, R. D. Brown, D. C. Kem and J. L. Brown, *Clin. Pharmac. Ther.* **33**, 741 (1983).
25. M. A. Ondetti and D. W. Cushman, *CRC Crit. Revs. Biochem.* **16**, 381 (1984).
26. N. C. Johnson, *Proc. Soc. exp. Biol. Med.* **108**, 518 (1961).
27. J. A. Halsted and J. C. Smith, *Lancet* 7642, 322 (1970).
28. W. R. Beisel, R. S. Pekarek and R. W. Wannemacher, in *Trace Elements in Human Health and Disease,* Vol. I (Eds. A. S. Prasad and D. Oberleas), p. 87. Academic Press, New York (1976).
29. J. D. Bogden, in *Zinc in the Environment* (Ed. J. O. Nriagu), p. 138, John Wiley, New York (1980).
30. H.-P. Roth and M. Kirchgessner, *Archs. Tierernährung* **24**, 283 (1974).
31. H. Y. T. Yang and E. G. Erdös, *Nature, Lond.* **215**, 1402 (1967).
32. W. Soudijn, *Pharm. Weekblad Sci. Edition* **4**, 154 (1982).
33. D. M. Gross, C. S. Sweet, E. H. Ulm, E. P. Backlund, A. A. Morris, D. Weitz, D. L. Bohn, H. C. Wenger, T. C. Vassil and C. A. Stone, *J. Pharmac. exp. Ther.* **216**, 552 (1981).
34. R. A. Skidgel, A. R. Johnson and E. G. Erdös, *Biochem. Pharmac.* **33**, 3471 (1984).