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COBALT-INDUCED ALTERATIONS IN PLASMA PROTEINS, PROTEASES AND KININ SYSTEM OF THE RAT

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Abstract—Rats receiving an injection of cobalt (Co) showed marked changes in plasma kininogen (Bradykininogen, BKG) levels, and alterations in arginine-esterase and bradykininase activity. A significant increase in BKG concentrations was seen at 4 and 24 hr after Co treatment, with maximum titers occurring 4 hr after treatment. Plasma kallikrein activity was significantly increased between 1 and 24 hr after Co treatment. Co initiated a decrease in plasma kininase activity, with minimum activity occurring 8 hr after treatment. Plasma protease showed peak activity approximately 8 hr after Co administration. However, Co added *in vitro* failed to enhance the proteolytic activity of rat plasma from normal as well as cobalt-treated rats. Plasma protein concentrations were significantly increased above control levels at 18 and 24 hr after cobalt treatment. These observations would seem to suggest that Co, which is known to initiate a state of histotoxic (tissue) hypoxia, might be triggering a mechanism(s) responsible for the changes seen in the plasma.

THE FACT that cobalt produces a polycythemia by initiating a state of histotoxic (tissue) hypoxia has been well documented.[1, 2] However, the precise mechanism by which cobalt exerts its effects is not yet clear. Levy *et al.*[3] and Yastrebov[4] have demonstrated cobalt to inhibit certain oxidative enzymes of the rat kidney *in vitro*. This observation was shown to be associated with decreased respiration and a fall in oxidative phosphorylation. We have shown, along with other investigators, that cobalt caused an increase in plasma erythropoietin (Ep) production.[5–7] Ep is a hormone believed to be produced by the interaction of a renal enzyme and a plasma α_2-globulin.[8–10] The newly formed Ep proceeds to stimulate erythropoietin-responsive cells in the bone marrow with the subsequent proliferation of new red blood cells.

Bradykinin is a 9-amino acid polypeptide produced by the action of an enzyme (kallikrein, kininogenase) on an α_2-globulin (bradykininogen, kininogen, kallidinogen) in the plasma.[11] Rocha e Silva[12] suggested the name "kinin hormones" for this class of polypeptides which have no specialized gland of secretion, being released from inactive precursors in plasma and most tissues including the intestinal tract and central nervous system.

In that the substrate for bradykinin formation and Ep production is an α_2-globulin, and because we have recently suggested that bradykininogen might serve as a

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substrate for Ep production after cobalt treatment,[5] the studies presented here represent an evaluation of the effects of cobalt on several parameters of the plasma kinin system as it exists in the rat.

MATERIALS AND METHODS

Animal experiments. Male Sprague-Dawley rats (250–350 g) received an injection of cobaltous chloride-hexahydrate (250 μmoles/kg, s.c.). The animals were sacrificed at 1, 4, 8, 12, 15, 18, 24, 48 and 72 hr after treatment. Rats receiving only solvent were sacrificed at the same time intervals and served as controls. At each time period, the rats were bled via aortic puncture with a heparinized plastic syringe. The blood was pooled in plastic centrifuge tubes which contained several drops of heparin (40 units/ml). Plasma was prepared by centrifuging the whole blood at 2000 rev/min for 10 min in an International centrifuge. The plasma was separated from the packed cell volume with a plastic syringe, stored in plastic vials, and frozen until used. Care was taken to avoid contact between the plasma and glass, in that glass is a known activator of kinin formation.[13]

Determination of bradykininogen in plasma. The plasma bradykininogen (BKG) was estimated by the amount of bradykinin liberated from denatured plasma by an excess of trypsin, according to the method of Diniz and Carvalho.[14] This method was employed in our laboratory, because we were concerned with the evaluation of plasma in terms of its BKG content. It has been indicated that the best method for quantification of total plasma kininogen is the method we employed in our studies.[15]

Biological assays. Bioassay of plasma kinin activity was performed on a segment of an isolated uterus preparation mounted in a muscle bath (10 ml) containing de Jalon solution at 30° and bubbled continuously with oxygen. The uterine preparation was taken from rats (200–225 g) which had received an intraperitoneal injection of stilboestrol (0·1 mg/kg) on each of the 2 days preceding the experiment. A log dose–response curve was calculated for each preparation. Kinin activity in the samples tested was converted to bradykinin equivalents. In some cases, the data were expressed as a percentage of control (kinin activity in plasma from untreated rats).

Bradykininase activity. Blood samples of 0·5 ml were employed for determination of plasma kininase activity. The plasma samples were incubated with 250 ng of bradykinin bitartrate in 1·0 ml of 0·1 M sodium phosphate buffer, pH 7·5, at 37° for 15 min. The reactions were terminated by immersing the incubate in a 100° water-bath for 2 min. The mixture was diluted with 1 ml of glass-distilled water, and 0·5 ml (0·1 ml plasma) of the mixture was assayed for bradykinin activity by employing the rat uterus bioassay. The response of the rat uterus to the test sample was converted to ng of bradykinin from a dose–response curve of known concentrations of bradykinin. The kininase activity is expressed as ng of bradykinin destroyed/min per 0·1 ml of plasma. The substrate concentration (bradykinin) was in excess according to the defined experiment.

Esterolytic activity. The plasma esterase (arginine-esterase) activity was determined according to the method of Hummel.[16] The substrate employed in these experiments was α-*p*-toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME. HCl) (Sigma Chemical Co.).

Proteolytic activity. An estimation of the proteolytic activity in plasma from normal and cobalt-treated rats was carried out as follows: 1 g of calcium caseinate

(Hammersten) was dissolved in 100 ml (10 mg/ml) of 0·15 M sodium borate buffer, pH 8·0. This solution served as a stock supply and was stable at 0° for a period of 1 month. For use in the protease assay, the stock solution was diluted 10-fold with borate buffer (pH 8·0) to reach a final concentration of 1·0 mg casein/ml.

One milliliter of the casein substrate (1 mg/ml), pH 8·0, was added to 0·2-ml samples of the plasmas being tested for protease activity. The pH was adjusted to 7·3 and the reaction mixture was incubated at 25° for various time intervals. The reaction was stopped by immersing the incubation tubes in boiling water for 2 min, and then cooled to room temperature. In order to determine the degree of protease activity, the method of Habeeb[17] was employed. This procedure involves the use of 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) which reacts specifically and under mild conditions with the free amino groups of a protein, polypeptide or amino acid (liberated by proteolytic activity) to yield trinitrophenyl derivatives (TNP) which can be read spectrophotometrically at 340 nm. In these studies, a modified TNBS reaction was employed according to the method of Lin *et al.*[18] One milliliter of 0·1% TNBS and 1·0 ml of 4% $NaHCO_3$, pH 8·5, is added to the boiled reaction mixture. The samples are stirred and allowed to incubate in the dark for 90 min at 38°. At the end of this time period, 0·5 ml of 1 N HCl and 1·0 ml of a sodium dodecyl sulfate solution (SDS) (Fisher Scientific Co.) are added to the incubation mixture. The SDS solubilizes the protein and prevents its precipitation upon addition of the HCl. The absorbance at 340 nm was read against a suitable blank. Recordings for the protease and esterase experiments were made with a Beckman DB-G spectrophotometer.

Plasma protein. These determinations were made according to the biuret method of Gornall *et al.*,[19] employing crystalline bovine serum albumin as a standard.

The statistical evaluation of the data was made by the use of Dunnett's method for comparing several treatments with a single control (Fig. 2, 3, 5 and 6),[20] and Duncan's new multiple-range test (Fig. 1) for comparing each treatment mean with every other treatment mean.[21]

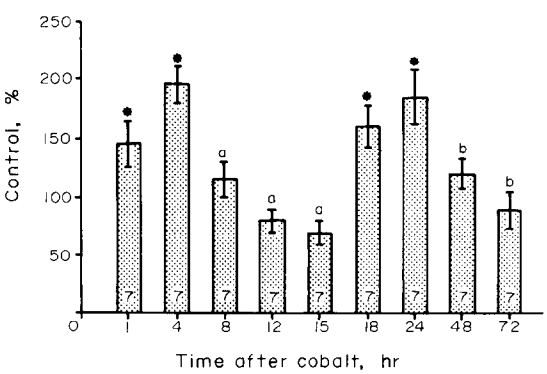

FIG. 1. Effect of cobalt on plasma bradykininogen levels in the rat. Each value represents the mean ± S.E.M. of seven experiments as indicated by the numbers at the bottom of the bars. Control value, 100 per cent (plasma bradykininogen levels in untreated rats, zero time interval). In these experiments, kinin levels are directly proportional to bradykininogen content in the quantitative procedure employed for determining bradykininogen levels in plasma. *, Significantly different ($P < 0·05$) from control; a, significantly different ($P < 0·05$) from 4-hr time interval; and b, significantly different ($P < 0·05$) from 24-hr time interval.

RESULTS

Effect of cobalt on kininogen (Bradykininogen, BKG) levels in rat plasma. The data in Fig. 1 illustrates the significant ($p < 0.05$) increase in circulating levels of BKG at 1 (40 per cent) and 4 hr (82 per cent) after a subcutaneous injection of cobaltous chloride-hexahydrate. The mean values were compared to each other as well as to control (zero time) values which were set at 100 per cent. There was a significant ($P < 0.05$) decrease in plasma BKG titers at 8 (39 per cent), 12 (55 per cent) and 15 (60 per cent) hr after cobalt administration, when these values were compared to the BKG levels at 4 hr. However, the BKG titers at these time intervals were not significantly different from control values. Cobalt caused a significant ($P < 0.05$) increase in BKG levels at 18 (70 per cent) and 24 (88 per cent) hr after treatment as compared to control levels. The BKG titers returned to control levels between 48 and 72 hr after cobalt administration. However, the BKG concentrations at 48- and 72-hr intervals were significantly less than the 24-hr values.

Effect of cobalt on plasma arginine-esterase levels. The substrate *p*-tosyl-L-arginine methyl ester (TAME) was used to quantitate plasma arginine-esterase activity. Activity is expressed as μM TAME split/60 min per 100 μl of plasma. Cobalt caused a significant ($P < 0.05$) increase in plasma arginine-esterase activity (Fig. 2) at 1, 4, 8, 12, 15, 18 and 24 hr after treatment with maximum activity occurring at the 4-hr time interval.

Plasma bradykininase activity. Kininase activity of the plasma (Fig. 3) was significantly ($P < 0.05$) decreased at 1, 4, 8, 12, 15 and 18 hr after cobalt administration. Figure 4 illustrates the results of a time study whereby a known concentration of bradykinin bitartrate (250 ng) was incubated with a sample of plasma from normal and cobalt-treated (8 hr) rats for 0, 5, 10 and 15 min. It is apparent that cobalt caused a decrease in the amount of kininase activity in the plasma from the cobalt-treated rats.

Plasma protease activity. Neutral protease activity (Fig. 5) was significantly ($P < 0.05$) increased at 1-, 4-, 8- and 12-hr intervals after cobalt treatment with maximum activity (130 ± 10 units/mg) occurring 8 hr after cobalt treatment as compared to control (zero time) values at 40 ± 5 units/mg.

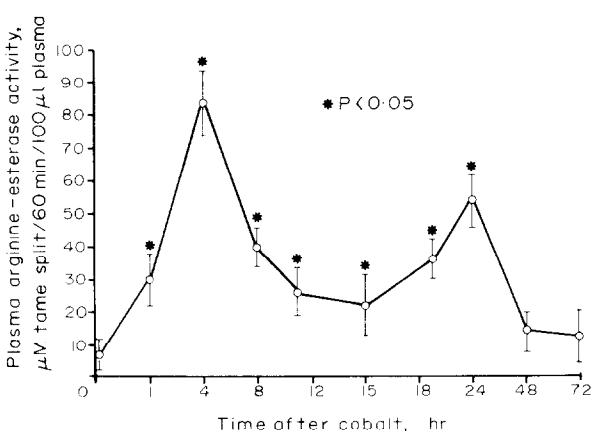

FIG. 2. Arginine-esterase activity in the plasma of cobalt-treated rats. Each value represents the mean + S.E.M. of six determinations. *. Significantly different ($P < 0.05$) from control values.

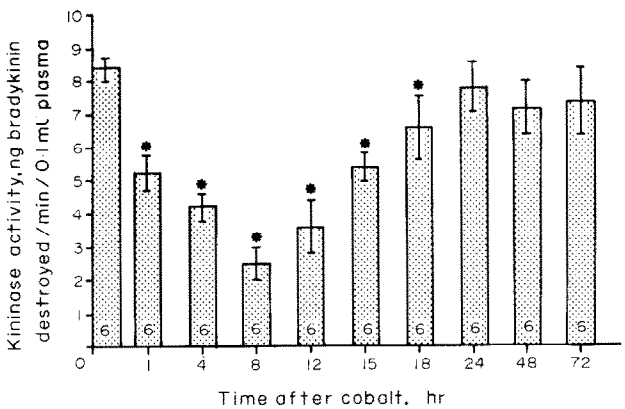

FIG. 3. Effect of cobalt on bradykininase activity in the plasma of cobalt-treated rats. The quantity of bradykinin remaining after a 15-min incubation of plasma with a known amount of bradykinin standard is an indication of bradykininase activity. Each value represents the mean ± S.E.M. using uterine muscle from six rats. *. Significantly different ($P < 0.05$) from control value.

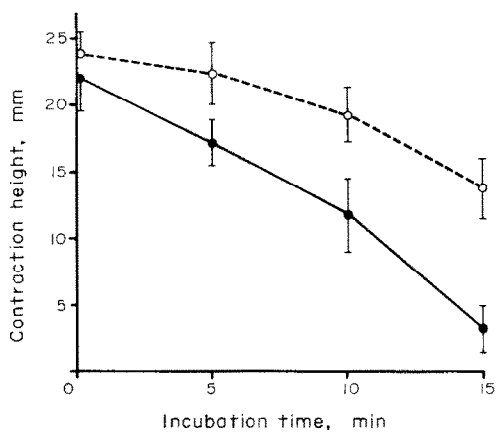

FIG. 4. Inactivation of bradykinin by plasma from cobalt-treated rats. Each value represents the mean ± S.E.M. of six experiments. Plasma from normal (●—●) and 8-hr cobalt-treated (○ ····○) rats was evaluated in this study. The incubations were stopped at 5-min intervals and aliquots were taken from the reaction mixture and evaluated for bradykinin activity with the rat uterus bioassay.

When cobalt (1×10^{-6}M) was added to an incubation mixture of plasma and casein substrate, the cobalt failed to produce any effect on the proteolytic activity of the plasma.

Effect of cobalt on plasma protein levels. Cobalt caused a significant ($P < 0.05$) increase in plasma protein concentration (Fig. 6) 18 and 24 hr after treatment. No change in protein titers was recorded at any other time interval.

DISCUSSION

In the present report, we have shown that cobalt stimulated an increase in plasma BKG levels with maximum titers occurring at the 4- and 24-hr time intervals. BKG

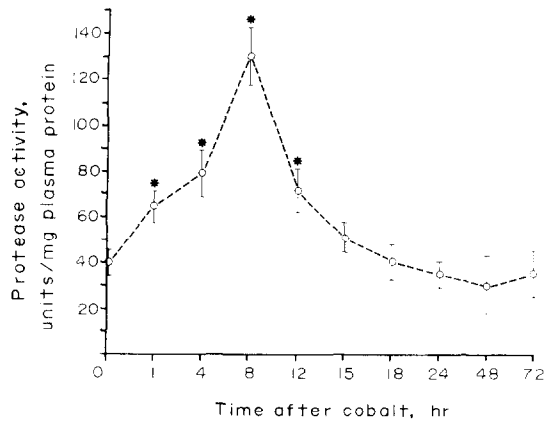

FIG. 5. Effect of cobalt on plasma protease activity in the rat. Each value represents the mean ± S.E.M. of seven experiments. *. Significantly different ($P < 0.05$) from control values.

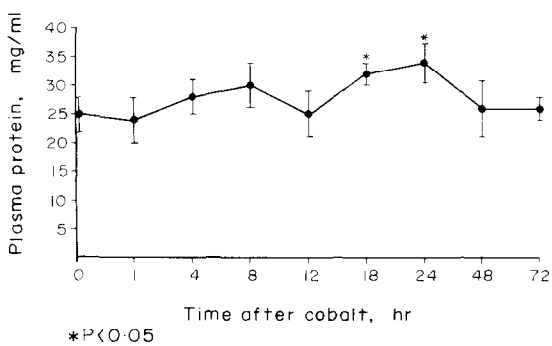

FIG. 6. Protein concentration in plasma from cobalt-treated rats. Each point represents the mean ± S.E.M. of four determinations. *. Significantly different ($P < 0.05$) from control.

levels decreased between 4 and 15 hr after cobalt administration. The return to control values occurred 72 hr after the rats received an injection of cobalt. The cyclical effect of cobalt on circulating BKG levels might reflect alterations in the synthesis or metabolism of BKG, and possibly its release from tissues which presumably have become hypoxic due to the actions of cobalt.

The highest levels of arginine-esterase activity are seen at 4 and again at 24 hr after cobalt treatment. The substrate employed to evaluate kinin-forming activity in plasma was TAME, and Hamberg and Rocha e Silva[22] have suggested that bradykinin-forming enzymes are capable of cleaving arginine esters. This relationship would seem to exist in the plasmas from rats treated with cobalt. However, it should be noted that the ester substrate used in our studies could conceivably be hydrolyzed by a variety of plasma enzymes.

Perhaps the point should be raised that cobalt, by inducing a state of histotoxic hypoxia, might in fact create an intracellular environment similar to that seen with ischemia. In this situation, a low pO_2 leads to a drop in the intracellular pH.[23] This situation causes the lysosomal membranes to labilize, with the eventual release of the enzyme content of the vesicular organelles.[24] These enzymes then proceed to par-

ticipate in the mechanism of cellular autolysis.[24-26] An enzyme, kininogenin, demonstrated to possess kinin-forming properties, has been localized in the lysosomal fraction of the rat kidney.[27] Cobalt might facilitate the release of kininogenin into the plasma where it could then exert its kininogenic role. An alternate possibility is that cobalt effects interaction between "intracellular" stores of BKG and lysosomal-bound kininogenin with the subsequent formation of the active polypeptide. The polypeptide would then be released from its tissue storage site into the circulation. Nustad and Rubin[28-30] localized kininogenase activity in the microsomal fraction of the rat kidney cortex. Whether or not kininogenase and kininogenin are one and the same has not as yet been made clear. However, it is interesting to note that both of these enzymes have been demonstrated to act upon arginine esters.

Cobalt exerted an inhibitory effect on plasma bradykininase activity. Bradykininase activity was low at a time when BKG levels were decreasing after the administration of cobalt. This observation is interesting in light of the fact that cobalt stimulates the activity of the two known plasma bradykininases (kininase I, kininase II). Carboxypeptidase N (kininase I) inactivates bradykinin by cleaving the carboxyl terminal Phe^8-Arg^9 bond of the nonapeptide.[31] The more recently discovered kininase II (peptidyl peptide hydrolase) acts as an endopeptidase by cleaving the bradykinin peptide at the Pro^7-Phe^8 linkage.[32] The kidney cortex contains three enzymes capable of inactivating bradykinin: a prolidase (imidopeptidase), a carboxypeptidase (peptidyl-amino acid hydrolase) and an endopeptidase (peptidyl peptide hydrolase),[33, 34] also referred to as peptidase P. This latter enzyme has been found to be localized in the microsomal fraction of the kidney, and it is similar in action to the plasma bradykininase –kininase II. The kininase activity described in this paper was studied at pH 7·5. Kininase I, kininase II and the kidney kininases have been demonstrated to act at or near this pH value. Bradykininase activity of splenic origin has been demonstrated, but these enzymes act optimally in acid pH ranges. It is possible, therefore, that we failed to pick up this activity in the plasma, if it was indeed present. One might expect bradykininase activity to increase at a time when BKG levels were falling, but cobalt may stimulate the action of a humoral factor(s) which might be responsible for the fall in BKG titers at the observed time intervals. However, if cobalt does stimulate the development of an ischemic condition, it is possible that several of the lysosomal bound enzymes might serve as agents of kinin inactivation. It is possible that these proteases might act in a kinin-inactivating capacity if they were released from their tissue sites into the circulation. Several of these hydrolases (cathepsins) have been demonstrated to inactivate bradykinin *in vitro*.[35-37] Indeed, these enzymes might act upon the BKG in the tissue, thus explaining the decrease in circulating titers.

Neutral protease activity in rat plasma was enhanced after cobalt treatment. It is interesting to note that protease activity is significantly elevated (4–8 hr) above control levels at a time when BKG titers are on the decline (4–15 hr) post cobalt. This increase in plasma proteolytic activity might explain the decreases in BKG levels.

Cobalt is known to initiate a histotoxic hypoxia followed by a polycythemia,[1, 2] and red blood cells contain a carboxypeptidase which will hydrolyze bradykinin.[38] In addition, quite often erythrocytosis is accompanied by a leukocytosis. Leukocytes, particularly the polymorphonuclear (PMN) variety, have been demonstrated to contain kininases and a protease(s) which act at a neutral pH.[39-41] Neutral protease

activity has been reported in spleen[42, 43] as well as liver,[44] and several investigators have indicated that the neutral proteases will attack casein,[40, 44–46] the substrate employed in our studies. Bradykinin has been demonstrated to induce a leukocytosis,[47] and if increased levels of BKG act to stimulate a leukocytotic condition, it may very well be that the enzyme content of the erythrocytes and leukocytes serve to destroy the circulating kinin and kinin-precursor.

Erdös *et al.*[31, 38] have shown cobalt to enhance the activity of plasma kininases but block kininase activity in the red blood cell. The tissue kininases play a more important physiological role than do the erythrocyte kininases; it is doubtful, therefore, that the fall in plasma kininase activity after cobalt administration was due to cobalt inhibition of erythrocyte kininase activity. We have demonstrated that cobalt has no effects *in vitro* on plasma protease activity in the rat.

In summary, the data in this report indicate that the metallic salt, cobaltous chloride-hexahydrate, has marked effects on plasma proteins, proteases and kinin system of the rat. Cobalt is unique in that it is the only metal known to stimulate red blood cell production. It is possible that the process by which cobalt stimulates erythropoiesis might be associated with the findings revealed by studies reported in this manuscript.

REFERENCES

1. J. M. ORTEN, F. A. UNDERHILL, E. R. MUGRAGE and R. C. LEWIS, *Proc. Soc. exp. Biol. Med.* **29,** 174 (1931).
2. K. WALTNER and K. WALTNER, *Klin. Wschr.* **8,** 313 (1929).
3. H. LEVY, V. LEVISON and A. L. SCHADE, *Archs. Biochem.* **27,** 34 (1950).
4. A. P. YASTREBOV, *Fedn. Proc. (Trans. Suppl.)* **25,** T630 (1966).
5. R. J. SMITH and J. F. CONTRERA, *Proc. Soc. exp. Biol. Med.* **141,** 895 (1972).
6. E. GOLDWASSER, L. O. JACOBSON, W. FRIED and L. PLZAK, *Blood* **13,** 55 (1958).
7. G. M. RODGERS, W. J. GEORGE and J. W. FISHER, *Proc. Soc. exp. Biol. Med.* **140,** 977 (1972).
8. A. S. GORDON, *Physiol. Rev.* **39,** 1 (1959).
9. J. F. CONTRERA and A. S. GORDON, *Science, N.Y.* **152,** 653 (1966).
10. J. F. CONTRERA, A. S. GORDON and A. H. WEINTRAUB, *Blood* **28,** 330 (1966).
11. E. WERLE, in *Polypeptides which Stimulate Plain Muscle* (Ed. J. H. GADDUM), pp. 20–7. Livingston, Edinburgh (1955).
12. M. ROCHA E SILVA, in *Symposium on Bradykinin and Vaso-dilating Polypeptides*, p. 177. First International Pharmacological Meeting, Stockholm. Pergamon Press, Oxford (1962).
13. D. ARMSTRONG, J. B. JEPSON, C. A. KEELE and J. W. STEWART, *J. Physiol., Lond.* **129,** 80 (1955).
14. C. R. DINIZ and I. F. CARVALHO, *Ann. N.Y. Acad. Sci.* **104,** 77 (1963).
15. F. G. ERDÖS, in *Handbook of Experimental Pharmacology* (Ed. E. G. ERDÖS), Vol. 25, p. 65. Springer Verlag, New York (1970).
16. B. C. W. HUMMEL, *Can. J. Biochem. Physiol.* **37,** 1393 (1959).
17. A. F. S. A. HABEEB, *Analyt. Biochem.* **14,** 328 (1966).
18. Y. LIN, G. E. MEANS and R. F. FEENEY, *J. biol. Chem.* **244,** 789 (1969).
19. A. G. GORNALL, C. J. BARAWILL and M. DAVID, *J. biol. Chem.* **177,** 751, (1949).
20. C. W. DUNNETT, *J. Am. statist. Ass.* **50,** 1096 (1955).
21. R. G. D. STEEL and J. H. TORRIE, in *Principles and Procedures of Statistics*, p. 107. McGraw-Hill, New York (1960).
22. U. HAMBERG and M. ROCHA E SILVA, *Experientia* **13,** 489 (1957).
23. H. BEAUFAY and C. DEDUVE, *Biochem. J.* **73,** 604 (1959).
24. C. DEDUVE and H. BEAUFAY, *Biochem. J.* **73,** 610 (1959).
25. G. WEISSMANN, *New Engl. J. Med.* **273,** 1084 (1965).
26. M. RICCIUTTI, B. SCHERLAG, E. STEIN and A. DAMATO, *Clin Res.* **26,** 245 (1968).
27. I. F. CARVALHO and C. R. DINIZ, *Ann. N.Y. Acad. Sci.* **116,** 912 (1964).
28. K. NUSTAD, *Br. J. Pharmac. Chemother.* **39,** 73 (1970).
29. K. NUSTAD, *Br. J. Pharmac. Chemother.* **39,** 87 (1970).
30. K. K. NUSTAD and I. RUBIN, *Br. J. Pharmac. Chemother.* **40,** 326 (1970).

31. E. G. ERDÖS, in *Metabolic Factors Controlling Duration of Drug Action* (Eds. B. B. BRODIE and E. G. ERDÖS), p. 159. Pergamon Press, Oxford (1962).
32. H. Y. T. YANG and E. G. ERDÖS, *Nature, Lond.* **215,** 1402 (1967).
33. E. G. ERDÖS, *Adv. Pharmac.* **4,** 1 (1966).
34. E. G. ERDÖS and H. Y. T. YANG, in *Hypotensive Polypeptides* (Eds. E. G. ERDÖS, N. BACK and F. SICUTERI), p. 235. Springer Verlag, New York (1966).
35. L. M. GREENBAUM and K. YAMAFUJI, *Life Sci.* **4,** 657 (1965).
36. L. M. GREENBAUM, K. YAMAFUJI and K. S. KIM, in *International Symposium on Vaso-Active Polypeptides: Bradykinin and Related Kinins* (Eds. M. ROCHA E SILVA and H. A. ROTHSCHILD), p. 21. Third International Pharmacological Congress, Sao Paulo (1966).
37. L. M. GREENBAUM and K. YAMAFUJI, *Br. J. Pharmac. Chemother.* **27,** 230 (1967).
38. E. G. ERDÖS, A. G. RENFREW, E. M. SLOANE and J. R. WOHLER, *Ann. N.Y. Acad. Sci.* **104,** 222 (1963).
39. A. JANOFF and J. D. ZELIGS, *Science, N.Y.* **161,** 702 (1968).
40. M. A. STILES and J. FRAENKEL-CONRAT, *Blood* **32,** 119 (1968).
41. L. M. GREENBAUM and K. S. KIM, *Br. J. Pharmac. Chemother.* **29,** 238 (1967).
42. J. LOSPALLUTO, K. FEHR and M. ZIFF, *J. Immun.* **105,** 886 (1970).
43. J. LOSPALLUTO, K. FEHR and M. ZIFF, in *Tissue Proteinases* (Eds. A. J. BARRETT and J. T. DINGLE), p. 263. North-Holland, Amsterdam (1971).
44. P. BOHLEY, J. KIRSCHKE, J. LANGNER, S. ANSORGE, B. WIEDERANDERS and H. HANSON, in *Tissue Proteinases* (Eds. A. J. BARRETT and J. T. DINGLE), p. 187. North-Holland, Amsterdam (1971).
45. L. A. MOUNTER and W. ATTYLH, *Blood.* **14,** 52 (1960).
46. J. FREI, C. L. BOREL, G. HORVATH, B. CULLITY and A. VANNOTH, *Blood* **18,** 371 (1961).
47. S. S. CARDOSO, D. S. AMORIM, A. D. NOGUEIRA, J. A. ALENCAR, A. P. CORRADO and S. M. REGO, in *International Symposium on Vaso-Active Polypeptides: Bradykinin and Related Kinins* (Eds. M. ROCHA E SILVA and H. A. ROTHSCHILD), p. 263. Third International Pharmacological Congress, Sao Paulo (1966).