Biochemical Pharmacology Vol 27 pp 2829–2834
Pergamon Press Ltd 1978. Printed in Great Britain

KININASES AND CONVERTING ENZYME IN HUMAN PLACENTA

ANDRZEJ LITOROWICZ and MICHAŁ MAŁOFIEJEW
Institute of Obstetrics and Gynaecology, Department of Physiology of Smooth Muscles of Uterus, Medical School, ul. M. Curie- Skłodowskiej 24 a, 15-276 Białystok, Poland

(*Received* 6 *September* 1977; *accepted* 30 *March* 1978)

Abstract—Homogenized tissue of the human placenta is capable of inactivating bradykinin. Ultracentrifuging of the homogenate has shown that the highest activity cosediments with ribosomal fraction. Partial purification of the enzyme by filtration on Sephadex G-200 has been performed. Three protein peaks of kininase and converting activities were obtained. The optimum pH of kininase activity of placenta was determined. An attempt to determine molecular weight was made with the use of Sephadex G-200.

Presented results indicate that the placental tissue of a normal woman produces enzymes with kininase activity and activity converting angiotensinase I to angiotensinase II, which may have significance in mechanisms of the local autoregulation of the blood flow.

Kininases constitute a natural protection of the body against excessive action of kinins. These enzymes disrupt peptide bonds of the kinin chain. Severance of whichever of the bonds causes an inactivation of the kinin molecule. Most important kininases are: kininase I breaking off C-terminal arginin and kininase II breaking off C-terminal dipeptide Phe-Arg, having also the activity of a converting enzyme [4, 5].

Tissues of the reproductive organ participate actively in plasma kininogenesis as well as in inactivation of kinins. It has been shown for example that the extract from the human placenta contains kinin forming activity i.e. it has enzymes that release kinins from plasma kininogen [13]. It has been shown too, that aminopeptidases occur in the placenta and that they transform relatively poorly active kallidin to bradykinin [18], as well as low molecular substances of a polypeptide character, blocking the influence of bradykinin on smooth muscles [11].

This work reports investigations of the substance occurring in the placenta, which causes bradykinin inactivation and conversion of angiotensin I to angiotensin II.

MATERIALS AND METHODS

Reagents

1. Sephadex G-25; G-200 (Pharmacia); 2. Bradykinin (BRS-640, Sandoz and Calbiochem); 3. (Asp^1-Ile^5) Angiotensin I (Schwartz–Mann); 4. Angiotensin II (Ciba); 5. Blue Dextran 2000 (Pharmacia); 6. Aquacid II (Calbiochem); 7. Pepsin (Serva Entvicklungslabor); 8. L-Arginine (Reanal, Hungaria); 9. DL-Arginine (Reanal, Hungaria); 10. Human Albumin (Bio.-Med. Poland); 11. Phsopho-glycero-aldehydo-dehydrogenase (Koch–Light Lab.); 12. Human Transferine (Bio.-Med., Poland); 13. EACA (Chemapol., Czechoslovakia); 14. Tris (hydroxymethyl methylamine) (Koch–Light Lab.); 15. Silicon (Clay Adams); 16. $CoCl_2 \times 6H_2O$ (P.O.Ch., Poland); 17. Na_2EDTA (P.O.Ch., Poland); 18. Sodium desoxycholate (Polfa, Poland); 19. Phosphate buffer pH 6.8 (Chemicals—P.O.Ch., Poland); 20. Ringer-Tyrod's solution; 21. Dialyzing membranes (C. H. Dexter).

Material

Placentae, obtained from women with the normal course of pregnancies resulting in normal delivery, were taken for investigations. The placentae immediately after delivery were placed into a container with ice-cold physiologic NaCl solution. Into the umbilical arteries canules were inserted and connected to a reservoir filled with cold physiologic NaCl solution and the placenta was washed out under the pressure of an 80 mm water column until the blood was fully removed. Washed out lobes were separated from the placenta through its full thickness, fetal membranes and larger blood vessels were removed and then placental tissue was dried on filter papers.

Methods

Fractionation of human placental homogenate. Fractionation was performed based on Schneider's method [16]. Devoid-of-blood placental lobes were frozen in dry-ice. After thawing, the tissue was cut into small pieces and then mixed in the ratio: 10 g of the tissue with 50 ml of the solution 0.25 M sucrose and homogenized for 2 min in a Servall Omni-Mixer. Following preliminary centrifuging of the filtrate, fractionation was carried out using a centrifuge (Servall) and ultracentrifuge (MSE). The following fractions were obtained:

1. 755 *g*—10 min nuclei and destroyed cells;
2. 2500 *g*—10 min—mitochondria;
3. 15,000 *g*—20 min—lysosomes;
4. 60,000 *g*—60 min—polysomes;
5. 150,000 *g*—30 min—ribosomes;
6. Supernatant.

To identify cell organelles, each obtained fraction was examined in the electron microscope EM-100 (Philips). The material was placed on Formwar-carbon nets, and stained negatively according to

Horne's method [7]. All the fractions but the last filtrate were treated with ultrasounds (MSE, 400 W, 5 min) in order to break up organelles and then the content of protein and kininase activity were determined.

Preparation of the material for purification. Placental tissue was placed in phosphate buffer 0.02 M pH 8.3 + 0.05 M NaCl/1 and homogenized. The homogenate was filtered through a nylon sifter and then centrifuged for 60 min using 60,000 *g* acceleration. The obtained material, containing polysomes and ribosomes and so-called soluble fraction, was saturated with sodium desoxycholate up to a final concentration of 0.26 per cent. After one hour mixing at the temperature of 4°, desoxycholate was removed by filtering on gel Sephadex G-25. In the preparation, the protein content, kininase activity and converting activity were determined. Water extract was concentrated by means of aquacid in dializing bags.

Molecular filtration. Inner surface of the glass column (2.2 × 45 cm) was covered with silicon. Sephadex G-200 was used, applying the flow as phosphate buffer 0.02 M pH 8.0 adding 0.05 M NaCl/1 from the bottom of the column to the top, at the rate of 10 ml/hr. Protein in fractions was determined spectrophotometrically. At the same time, kininase, converting and angiotensin activities were estimated. The material for investigations was stored at −10°. At particular stages of the preparation, the protein content was estimated by the Lowry method [12].

Biological measurements. The experiments were performed on isolated small intestines of guinea pigs and isolated uterine horns of estrogenized Wistar rats. Measurements were done with the isotonic method of Magnus in the oxygenated Ringer–Tyrode's solution. The temperature of the solution for intestines was 37°, for isolated uteri 18°. The uteri at that temperature did not show any spontaneous contractile activity.

Measurement of kininase activity. The following systems were used to measure kininase activity from particular fraction of chromatography: 0.2 ng of bradykinin +0.2 ml of the investigated sample were incubated in silicon tubes at 37° for 30 min. The investigated material for incubation was diluted 1:100. To define activity in initial as well as in end products of purification, the same system of the test as before, but different times of incubation, were used. After a defined time of incubation, the samples were fixed by boiling for 10 min and then were frozen. The quantity of bradykinin inactivated by the investigated preparation was estimated biologically. The quantity of inactivated bradykinin was calculated by comparing the contraction induced by adding to an organ-bath the investigated sample with the dose–effect curve [17]. Bradykinin used for control samples was stored in conditions identical with those of investigated samples.

The calculated quantity of inactivated bradykinin was referred to the protein content in a given sample.

Measurement of angiotensinase activity. The measurement of angiotensinase activity was performed on the basis of the Khairallach's work [8]. 0.02 ng of angiotensin II +0.2 ml of investigated sample were incubated for 30 min at 37°. The reaction was being broken by 10 min boiling. The degree of angiotensin II inactivation was measured on the isolated rat uterus.

Measurement of angiotensin I conversion to angiotensin II. The measurement of conversion ability of the investigated preparations was done in the following systems: 10 ng of angiotensin I +0.2 ml of investigated sample were incubated at room temperature [25°] in the medium containing NaCl in concentration of 0.15 M/1 and after a defined time of incubation the investigated sample without fixation was placed in an organ-bath with the isolated rat uterus. The degree of conversion was estimated by comparing the contraction induced by the investigated sample with the contraction induced by a known dose of angiotensin II.

The above procedure did not give results when, for incubation with angiotensin I, an initial material was used even partly purified. Angiotensinases contained in preparations quickly decomposed formed angiotensin II and the demonstration of conversion became impossible.

Basing on data from the literature [9, 19] it results that the removal of Ca ions from the medium suppresses the activity of angiotensinases but has no influence on converting activity, while the introduction of cobalt ions causes only a partial return of angiotensinase activity (about 40 per cent) and the activation of converting enzyme even to 140 per cent. An exchange of ions was performed in the investigated preparation. At the first stage, the investigated preparation was dialyzed with reference to Na_2EDTA at the 10^{-4} M/1 concentration to remove metal ions from the enzymatic medium. At the next stage, this preparation was dialyzed against $CoCl_2$ solution at the 10^{-4} M/1 concentration. These procedures enabled us to show the presence of converting enzyme in preparations from chromatography; however they turned out ineffective in the face of entirely non-purified water extracts from the placental tissue.

Determination of optimum pH of the placental kininase activity. Partly purified enzyme was incubated with substrate in media at pH 6.0, 7.0, 8.0, 9.0, for 30 min; the incubation was by boiling for 10 min. The activity was measured biologically. As a control the substrate (bradykinin) was incubated without enzyme in the same conditions.

The influence of kininase inhibitors on the investigated enzyme activity. The enzyme was incubated with some kininase I inhibitors at 37° for 30 min and then the substrate was added to the system and incubated for further 30 min at the same temperature. The incubation was interrupted by boiling for 10 min, the activity being measured with a biological test. An identical system with no inhibitor was used.

The test to determine placental kininase molecular weight. Active fractions obtained from molecular filtration were used for investigations. The column 2.2 × 45 cm filled with Sephadex G-200 was applied. Elution was carried out at the rate of 10 ml/hr. The volume of the introduced sample into the column was 1 ml, the volume of the pooled fraction was 3.6 ml.

The following standard proteins were used: cytochrome *C*, molecular weight 12,600; pepsin, molecular weight 35,000; bovine albumin, molecular weight 69,000; human transferine, molecular weight 90,000;

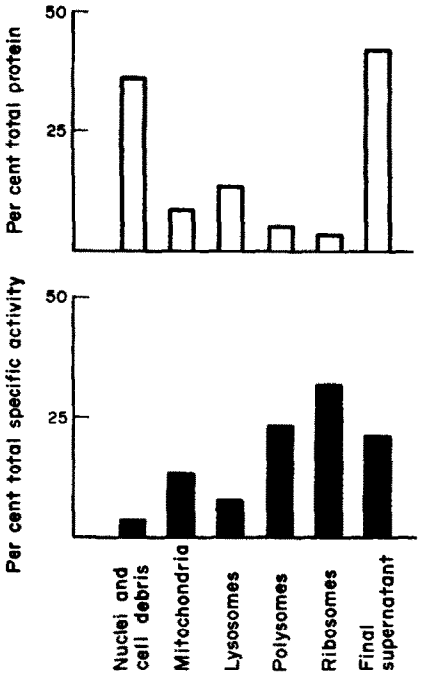

Fig. 1. Distribution of kininase activity in subcellular fraction of homogenates of human placenta.

phosphoglyceraldehyde dehydrogenase, molecular weight 140,000. Dextran Blue 2000 was used to determine the void volume of the column (V_0). In calculations, the coefficient of the relative eluation volume (V_e/V_0), recommended by Determan, was used [3].

RESULTS

Kininase activity of subcellular fractions. The highest activity was shown by the ribosomal fraction containing about 1 per cent of total protein and 35.5 per cent activity and by the polysomal fraction containing about 3.5 per cent of total protein and 23.5 per cent activity (Fig. 1).

Purification of substances with kininase activity. The removal of nuclei fragments of smashed cells and mitochondria from the preparation causes almost a three-fold increase in specific activity. Further a two-fold increase in the activity is noted after deoxycholate administration, filtration on coarse Sephadex G-25 and following concentration. In such a manner obtained preparation was filtered on the Sephadex G-200 column. It was found that fractions (I) 22–26, (II) 29–42, (III) 50–54 and (IV) 59–61 possess the capability to inactivate bradykinin (Fig. 2A). The activity of angiotensin conversion to angiotensin II (Fig. 2B) occurred in these fractions, too. The purification process performed so far is shown

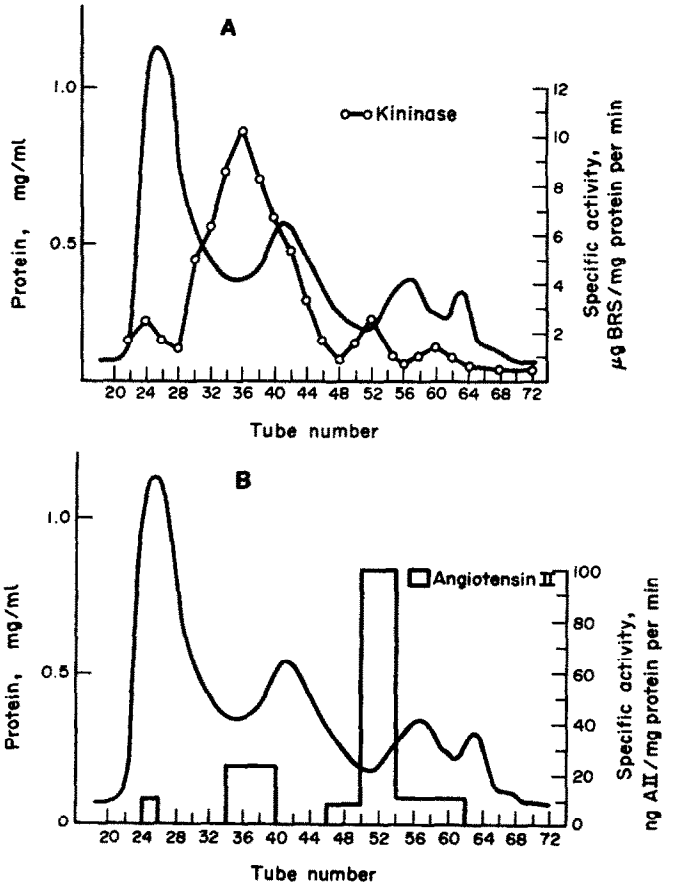

Fig. 2. Purification of placenta kininase by gel filtration on Sephadex G-200. Bed dimensions 2.2 × 45 cm. Flow rate: 10 ml/hr. Eluant: 0.02 M phosphate buffer, pH 8.0, and 0.05 M NaCl. The protein was detected spectrophotometrically. [Kininase activity was measured with BRS 640 on the isolated guinea pig intestinum —(A). Converting activity with (Asp^1-Ile^5) Angiotensin I on the isolated rat uterus—(B).

	Total protein (mg)	Total units (μg BRS /min)	Specific activity (μgBRS/mg per min)	Purific coeffic-ient
Homogenate	342.00	325.50	0.68	
Supernatant 20.400 x g-30 min	88.40	182.90	2.07	3
Supernatant (Deoxycholate, G-25, Agvacid II)	46.38	170.20	3.67	6.1
I fraction 22-26	12.96	32.40	2.50	3.7
II fraction 29-42	11.20	114.20	10.20	18.4
III fraction 50-54	1.32	2.57	1.95	2.8

Fig. 3. Purification of kininase from human placenta.

in Fig. 3. Homogenate centrifuging at the speed of 20,400 *g* for 30 min caused the loss of 75 per cent protein and a triple increase in specific activity compared to fresh homogenate.

The action of sodium dezoxycholate and then filtration on Sephadex G-25 coarse, concentration and centrifuging caused the loss of 50 per cent of protein from the previous stage and a further two-fold increase in activity. Continuation of the table represents comparison of the protein content and activity in fractions from chromatography on Sephadex G-200. Fractions 22–26 containing 28 per cent of protein introduced into the column, and 19 per cent of activity, show almost a four-fold increase in activity compared to the homogenate activity. Fractions 29–42 contain 24 per cent of protein and 67 per cent of activity and show more than 18-fold increase in activity. Fractions 50–54 contain 2.8 per cent of protein and 1.5 per cent of activity, an almost three-fold increase in activity compared to homogenate. Fractions 59–61 contain 0.7 per cent of protein and 0.8 per cent of activity and show practically no increase in specific activity. Angiotensinase activity was also determined in the fractions from chromatography. The activity is represented by a continuous line with circles in Fig. 4.

The curve profile, representing angiotensinase activity does not allow any decisive isolation to be made of active sites in the chromatogram. In fractions 28–32 slight activity occurs. Beginning occurs with two peaks visible in fractions 38–48. Angiotensinase activity has been calculated in ng of inactivated angiotensin II preparation with reference to the protein content in particular fractions.

Action of inhibitors. The effect of inhibitors capable of affecting inhibiting kininase I capacity (carboxypeptidase N) on the placental kininase activity was investigated. Besides Na_2EDTA, the following compounds were used: cadmium sulphate ($CdSO_4$), L-arginine, DL-arginine and epsilon-amino capronic acid (EACA). In our conditions only Na_2EDTA inhibits placental kininase activity.

Influence of pH on bradykinin inactivation affected by placental kininase. Two buffers were used: phosphate buffer 0.02 M at pH 6.0–8.0 and Tris–HCl buffer 0.02 M at pH 8.0–9.2. Most favourable to the reaction of placental kininase—bradykinin appeared to be the medium at pH about 8.0.

At extreme values, i.e. at pH 6.0 and 9.2, enzymatic activity was lower, in the medium of both the buffers (Fig. 5). The change of pH to 7.0 caused a weaker (by about 35 per cent) bradykinin inactivation, whereas at pH 6.0 bradykinin inactivation occurred only in about 20 per cent. On the side of alkaline reaction the pH shift to 9.0 caused a decrease in inactivation by 25 per cent. Further pH increase to 9.2 decreases inactivation by 40 per cent.

Determination of molecular weight of placental kininase by gel filtration on Sephadex G-200. Void volume (V_o) of the column is 83.4 ml. Individual values of the so called relative elution volume (V_e/V_o) of active protein obtained from chromatography were from fractions 22–26 I V_e/V_o = 1.04, from fractions 29–42 II V_e/V_o = 1.53. Figure 6 represents the mode of molecular weight calculation. Individual values of relative eluation volume of standard proteins were laid off on ordinate axis, their molecular weights were laid off on abscissa axis in the logarithmic scale. In the calculation of the linear dependence between these

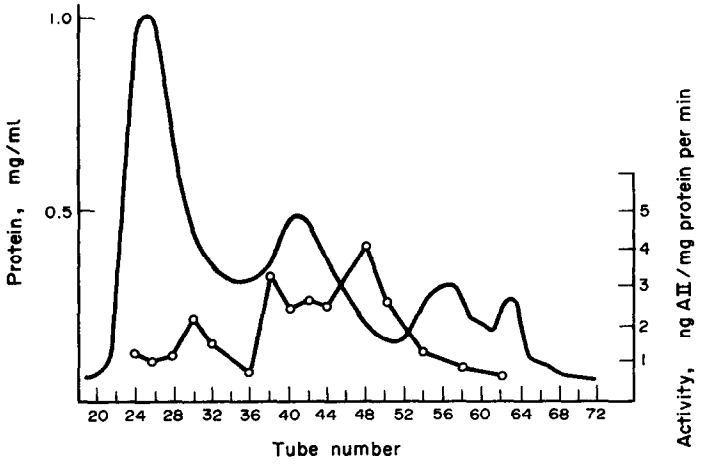

Fig. 4. Angiotensinase activity in the fraction of gel filtration on Sephadex G-200 (the same gel filtration as on Fig. 2).

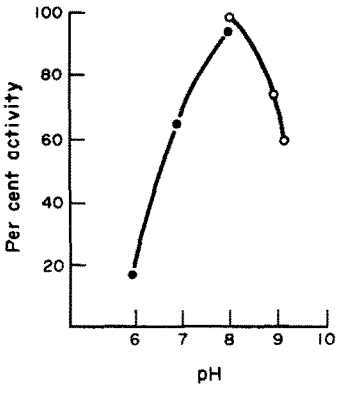

Fig. 5. Optimum pH determination of placental kininase activity. Left curve, phosphate buffer; right curve, Tris–HCl buffer. Molarity of buffers 0.02.

values, the method of least squares was used. Particular peaks of placental kininase activity correspond with the following values of molecular weight: I—479,000; II—159,000; III—24,300. Because of an extreme position of I and III values of the peak, they should be accepted as calculated with large approximation.

DISCUSSION

It has been found in this study that in the tissue homogenate of the womens placentae, beside angiotensinase activity, kininase activity and activity converting angiotensinase I to angiotensinase II.

Kininase activity sedimentates with both the ribosomal and polysomal fractions; it has also been found in supernatant. In the molecular filtration on Sephadex G-200, the mixture of ribosomal and polysomal fractions and supernatant underwent division into three peaks with kininase activity. In each of these peaks there was the activity converting angiotensin I to angiotensin II, too.

Using the methods recommended by Determan [3] we have approximately determined the molecular weight of the first peak as about 479,000, of the second peak as 150,000 and that of the third as 24,300. The molecular weight value of the first peak is approximate only, because it lies at the limit of distributive capability of the Sephadex used.

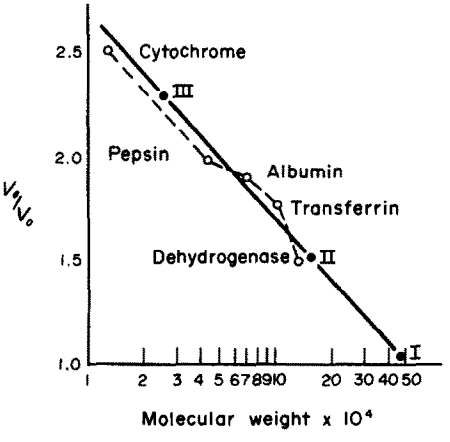

Fig. 6. Trial to determine molecular weight of placental kininase by gel filtration on Sephadex G-200.

With the use of inhibitors, such as EDTA, $CdSO_4$, L-arginine, DL-arginine and EACA, it results that in the placental tissue homogenates kininase II is found. Kininase II—converting enzyme shows optimum of action at pH 7.0–8.0; shifting towards acidic side of alkaline one causes rapid inactivation. The division of kininase activity in chromatography on Sephadex G-200 into three distinctly demarcated peaks shows that in the placental tissue different forms of the same enzyme are found, or different enzymes decomposing bradykinin and converting angiotensin I. Both the assumptions have real grounds since there are data concerning kininase II polymerization—a converting enzyme [15] as well as enzymes forming kininase II are known [2, 6]. The lack of a distinct division of angiotensinase activity may be caused by the presence in the placental tissue of various angiotensinases, also unspecified.

It is known that angiotensinase activity of plasma of healthy pregnant women increases with the age of pregnancy and decreases immediately following delivery [14]. The behaviour of the angiotensinase level is associated with the production of these enzymes by the placenta [5]. The plasma of pregnant women with hypertension shows significantly lower values of angiotensinase activity compared to that of healthy pregnant women [10]. These facts suggest that metabolic disturbances are responsible for some symptoms of gestosis.

The occurrence of the enzyme converting angiotensin I to angiotensin II and inactivating kininase in the human placenta may testify that the organ interferes with the local economy of active peptides within the sphere of the pregnant uterus, and it might, as with placental angiotensinases, participate in the systemic circulation.

However, determination of the real role of the placental converting enzyme requires further investigation.

Acknowledgements—We wish to thank Professor E. G. Erdös for his valuable methodical information and for delivery of angiotensin I preparation, and Mrs. R. Kozłowska for her technical assistance. This study was supported by the Pathophysiology Committee, Dept. VI, Polish Academy of Sciences.

REFERENCES

1. M. Berger and J. Langhans *Am. J. Obstet. Gynec.* **98**, 215 (1967).
2. R. Boucher, J. Asselin and J. Genest, *Circulation Res.* Vol XXXIV and XXXV, Suppl. 1–203 (1974).
3. H. Determan, *Gel Chromatography*, p. 107. Springer, Heidelberg (1969).
4. E. G. Erdös and H. Y. T. Yang, *Kininases* in *Handbook of Experimental Pharmacology* (Eds E. G. Erdös and A. F. Wilde), Vol. 25. p. 289. Springer, Heidelberg (1970).
5. E. G. Erdös, R. Igic, H. S. J. Yeh and K. Sorrels, *Identity of kininase II with an angiotensin I converting enzyme* in *Vasopeptides. Chemistry, Pharmacology and Pathophysiology* (Eds N. Back and F. Sicuteri), p. 149. Plenum Press, New York (1972).

6. A. Grandino and A. C. M. Paiva, *Biochim. biophys. Acta* **364**, 113 (1974).
7. R. W. Horne, Negative staining methods, in *Techniques for Electron Microscopy* (Ed. D. Kay) p. 325 Blackwell, Oxford. (1965).
8. P. A. Khairallah, F. M. Bumpus, I. A. Page and R. R. Smeby, *Science N.Y.* **140**, 672 (1963).
9. P. A. Khairallah and I. H. Page, *Biochem. Med.* **1**, 1 (1967).
10. R. Landesman, P. Biron, R. Castellanos and R. Russa, *Obstet. Gynec.* **22**, 316 (1963).
11. A. Litorowicz, *Ginek, pol.* **47**, 879 (1976).
12. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. M. Małofiejew and A. Litorowicz, *Ginek. pol.* **47**, 369 (1976).
14. G. Morandini and C. Magnoni, *Acta med. scand.* **185**, 311 (1969).
15. T. Nakajima, G. Oshima, H. S. J. Yeh, R. Igic and E. G. Erdös, *Biochim. biophys. Acta* **315**, 430 (1973).
16. W. C. Schneider, Fractionation of animal tissue cells, in *Cyril Long Biochemist Handbook* (1965)
17. I. Trautschold. Assay methods in the kinin system, in *Handbook of Experimental Pharmacology*, (Eds. E. G. Erdös and A. F. Wilde) Vol. 25, p. 52. Springer, Heidelberg (1970).
18. M. Uszynski and M. Małofiejew, *Biochem. Pharmac.* **20**, 3211 (1971).
19. H. Y. T. Yang, E. G. Erdös and Y. Levin *J. Pharmac. exp. Ther.* **177**, 291 (1971).