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DETERMINATION OF PLASMA KININ AND KININOGEN LEVELS IN MAN

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SUMMARY

1. A method for the estimation of free plasma kinin and kininogen is described, which is suitable for samples of blood taken in hospital.

2. The method permits the assay of very low levels of kinin and substantially eliminates errors due to the presence of amines and other interfering substances.

3. In normal subjects the mean value of free kinin is 2·8 ng (bradykinin equivalent)/ml. plasma, and 6·1 μg kininogen/ml.

4. In vasovagal fainting, carcinoid flush, and dumping syndrome, during the phase of peripheral vasodilatation, the free kinin exceeds 10 ng/ml., and is often in excess of 30 ng/ml.

5. Sudden release of free kinin is accompanied by a fall in kininogen level.

INTRODUCTION

Recent studies have shown that the group of plasma kinins must be added to the list of possible mediator substances formed or set free in tissues as the result of a wide variety of conditions, including heat injury (Edery & Lewis, 1963; Goodwin, Jones, Richards & Kohn, 1963; Rocha e Silva & Rosenthal, 1961), parasitic infection (Goodwin & Boreham, 1966), arthritis (Eisen & Keele, 1966; Goldfinger, Melman, Webster, Sjoerdsma & Seegmiller, 1964), pregnancy (Martinez, Carvalho & Diniz, 1962; Periti & Gasparri, 1966) and anaphylaxis (Brocklehurst & Lahiri, 1962; Cirstea, Suhaciu & Butculescu, 1966; Greef, Scharnagel, Lühr & Strobach, 1966).

Investigation of their possible role in clinical conditions demands close co-operation between the clinician and pharmacologist, since inexpert handling of samples will lead to wildly inaccurate results.

The levels of kinin normally present in circulating blood are close to the threshold of the most sensitive assay procedure, and the levels in chronic clinical conditions are not likely to be very greatly raised because of the short life of kinins *in vivo*. It is evident that both destruction and activa-

tion of kinins must be arrested immediately in samples of blood or any other biological material. This paper describes a reliable method of collecting samples which is acceptable to the clinician and avoids the need for immediate preparation for assay by the pharmacologist. The extraction procedure used allows the active kinins to be concentrated and removes substances which would be expected to interfere in the bio-assay. Both active kinin and the protein precursor(s) may be assayed in the same sample of blood. The methods have been developed from those described by Brocklehurst & Lahiri (1962) and Gaddum & Horton (1959). Their usefulness is illustrated by data relating to human blood samples.

METHODS

Collection of the sample

Six millilitres of blood is taken without anticoagulant into a sterile disposable polystyrene syringe, through a disposable siliconized needle. One millilitre is rapidly placed in a sequestrene tube (as used routinely in hospital) for haematocrit estimations, the remainder is immediately inactivated by forcibly ejecting it through the needle into 15 ml. of chilled ethanol in a stoppered glass centrifuge tube, which is then shaken to give adequate dispersion. The sample may then be stored at 4° C for at least 8 hr if necessary. The importance of clean venepuncture and of speed once the blood has been removed from the vein and until it has been inactivated cannot be too strongly emphasized.

Preparation and assay

The coagulated protein is scraped from the stopper and the sides of the centrifuge tube, and the sample is centrifuged hard (2000 *g* for 30 min). The supernatant contains the free kinin while the precipitate contains the precursor. The precipitate is washed with 10 ml. 80 % (v/v) ethanol and again centrifuged. The washings are added to the first supernatant and kept in ice.

Kininogen. The washed precipitate is suspended in 10 ml. 80 % ethanol, and placed in a boiling water-bath for 10 min to give complete denaturation. The suspension is centrifuged hard and the supernatant discarded. The precipitate is washed twice with 10 ml. distilled water, then suspended in 15 ml. 2·5 M-NaCl solution. The suspension is homogenized in a ground-glass blender (Loughborough Laboratory Apparatus Ltd, 20 mm diam.) and 0·2 ml. aliquots of the homogenate are incubated at 37° C for 30 min with pure crystalline trypsin 200 μg/ml. ('Tryptar', salt-free, Armour Ltd., Eastbourne) in 5 ml. of 0·02 M sodium phosphate buffer, pH 7·35. The trypsin is then inactivated by heating in a boiling water-bath for 10 min. The solutions are now ready for assay, but may be quickly deep-frozen in solid CO_2 and stored at $-20°$ C. Assay involves comparison with standard synthetic bradykinin using the isolated rat uterus bathed in oxygenated de Jalon solution at 33 °C in the presence of atropine 1 μg/ml. (similar to the method of Gaddum, Peart & Vogt, 1949). It is advisable to ensure that all the kininogen has been converted (i.e. that an excess of tryptic activity has been used in the experiment) by incorporating an internal check in each experiment. In the present work a laboratory standard kininogen was prepared from human plasma by the same ethanol and heat treatment used in the preparation of the whole blood samples. It was shown to be stable and free from kininase, and its maximum yield of kinin was determined. Samples of this substrate representing 0·1 ml. plasma were treated in the same way as the samples being assayed in each experiment, to ensure that the maximum yield of kinin had been obtained.

Freeze-dried (lyophilized) trypsin is hygroscopic, and to avoid possible loss of potency it should be distributed aseptically into small bottles containing enough for a single experiment (about 20 mg). These samples must be carefully dried *in vacuo* over $CaCl_2$ and the containers filled with dry nitrogen before being closed.

Fresh batches of trypsin should be tested for freedom from enzymes able to destroy bradykinin during incubation. The amounts and conditions stipulated for the splitting of kininogen are used, with 20 ng bradykinin in place of kininogen. It is necessary to run parallel tests without trypsin because absorption on to the tube. and destruction due to lack of sterility may result in a small loss.

Free kinin. The kinin-containing ethanol extract is placed in a boiling water-bath for 10 min to destroy any latent enzyme activity, and is then evaporated to dryness under reduced pressure. The sample is reconstituted in 11 ml. warm distilled water (60° C), saturated with sodium chloride and acidified to pH 1·5 with hydrochloric acid. This solution is then shaken with butanol in a separating funnel to extract the peptides using first 10 ml. and then 5 ml. of butanol. The butanol is evaporated without undue delay under low pressure, starting at about 15° C and rising to 50° C. The pressure is finally reduced to 0·1 mm mercury and the temperature raised to 80 °C for 5 min to remove the last traces of butanol. The samples are stored dry at −20° C until assay on the isolated rat uterus, in the presence of atropine, 1 μg/ml., and a 5-HT antagonist (bromolysergic acid diethylamide 0·5 μg/ml). The dry samples which contain some lipid are shaken with 2 ml. of warm de Jalon solution to give an opalescent mixture immediately before assay. At all stages following inactivation in ethanol, samples are kept at 4° C or less unless otherwise stated, and the time during which the free kinin is in aqueous solution is kept to a minimum. All aqueous solutions of kinin were handled with siliconized glassware.

All solvents were glass-distilled, and all reagents used were of Analar grade.

RESULTS

Assay. The isolated uterus from a rat in full oestrus is suitable for both kininogen and free kinin assays. The total amount of kininogen in blood is relatively large, and therefore changes may be less than 20 %. The rat uterus will easily detect changes of less than 10 % in the applied dose of bradykinin (Fig. 1). Free kinin changes, to be of interest, are generally much more than 100 %; however, the total quantities are very small. The rat uterus is the most sensitive known routine assay for kinin, and in addition is insensitive to histamine and the slow reacting substance of anaphylaxis.

Recovery and specificity. The extraction of kinin carried out as described gave a recovery of 81·5 % (S.D. = 8·0 %) in eight experiments in which synthetic bradykinin (50 − 250 ng) was added to the ethanol at the same time as 5 ml. of venous blood. The low pH required for optimum recovery of kinin during the butanol extraction also causes amines of high pK_a to be highly ionized, and to remain in the aqueous phase. The method will thus largely exclude the interfering amines histamine (pK_a = 9·7), adrenaline (pK_a = 9·9), and 5-HT (pK_a = 10·0). The recovery of histamine (50 μg) added to the ethanol at the same time as 5 ml. of venous blood and treated as described was 0·4 % (S.D. = 0·08 %) in four experi-

ments. The recovery of added 5-HT (500 μg) similarly treated was found to be 3·2 % (S.D. = 0·86 %) in five experiments. Potassium released from lysed blood cells might be expected to interfere with the bio-assays when low kinin concentrations are being measured. The butanol extraction was found to exclude 93·8 % (S.D. = 2·1 %) of the blood potassium estimated by flame photometry in five experiments.

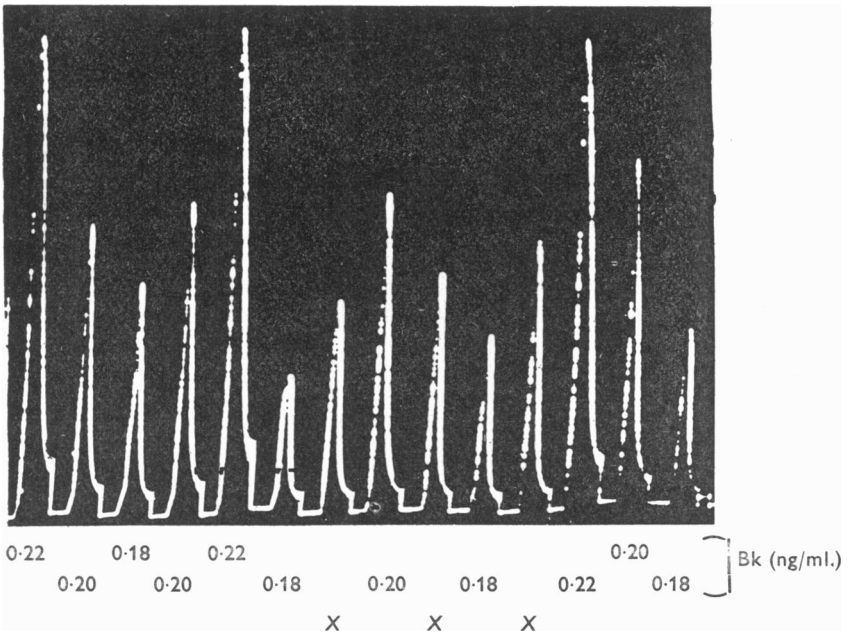

Fig. 1. Tracing from isolated rat uterus (in 4 ml. de Jalon solution, 33° C), showing a dose–response curve to bradykinin, and a typical assay. (Bk = standard bradykinin; X = sample being assayed.)

All other methods have used plasma for the evaluation of kininogen, the present method uses denatured whole blood and comparative studies were therefore necessary. Estimations of the plasma kininogen carried out with whole blood, and using the haematocrit value to calculate the volume of plasma used, gave values which were 88·7 % (S.D. = 5·8 %) of the value obtained in five experiments in which duplicate samples were processed using denatured plasma according to a method to be described by W. E. Brocklehurst & S. C. Lahiri (1967).

Experiments were also carried out to test the efficiency of enzymic inactivation by boiling ethanol. The inactivated whole-blood homogenate was tested for spontaneous kinin releasing activity in the absence of trypsin (four experiments), and for kininase activity (two experiments). The

homogenate did not contain significant amounts of either type of activity.

Normal values of plasma kininogen in venous blood. Blood from three groups of 'normal' subjects has been examined, a group of eleven elderly (61–85 years) convalescent women in hospital, six men (49–61 years) under similar conditions, and thirteen healthy active young men (20–30 years). The mean kininogen level for the women was 7·1 (S.D. = 1·1) μg bradykinin equivalent (Bk.eq)/ml. of plasma, for the older men it was 6·2 (S.D. = 1·1)μg Bk.eq/ml. plasma, for the young men the kininogen level was 5·1 (S.D. = 1·2)μg Bk.eq./ml. plasma. The mean value for the three groups considered together is 6·1 (S.D. = 1·4)μg Bk.eq./ml. plasma.

Normal free plasma kinin levels in venous blood. The normal (i.e. symptomless) level of free kinin in plasma was just above the threshold of detection for the method; occasionally no free kinin was detectable. In a group of sixteen men (20–60 years) a mean level of 2·8 (S.D. = 1·7)ng Bk.eq/ml. plasma was found.

Two healthy normal males (20 and 27 years) who underwent vasovagal fainting during blood sampling each had raised free kinin levels of 31·1 and 30·1 ng Bk.eq./ml. plasma respectively.

The release of plasma kinin in pathological conditions. As presented with clinical details elsewhere (Zeitlin & Smith, 1966), the methods described in this paper have been used to demonstrate a release of plasma kinin during flushing in patients having carcinoid tumours, and during flushing and hypotensive episodes in patients subject to the dumping syndrome.

Four carcinoid patients were examined after biochemical, histological and clinical investigations had confirmed that they were suffering from hydroxyindole-secreting carcinoid-like tumours. In these patients, the level of free kinin was directly related to the severity of flushing, the patient showing the most severe symptoms having the highest level of free kinin. The level of free kinin was found to increase dramatically with the onset of flushing and to return to near normal at its cessation. There was a fall in kinin precursor level during the carcinoid syndrome and the kinin activity was shown to be bradykinin-like by parallel bio-assay on the rat uterus and isolated rat duodenum.

Four partially gastrectomized patients suffering from the dumping syndrome were examined. The syndrome took the form of intestinal symptoms followed by syncope-like hypotensive flushing episodes, which could be provoked by ingestion of hypertonic (20 %) glucose solution. The vasomotor symptoms were always accompanied by dramatic increases in free plasma kinin, and a concomitant fall in kinin precursor level (Fig. 2 (i) and (ii)). The free kinin was destroyed by chymotrypsin and shown to be bradykinin-like by parallel bio-assay.

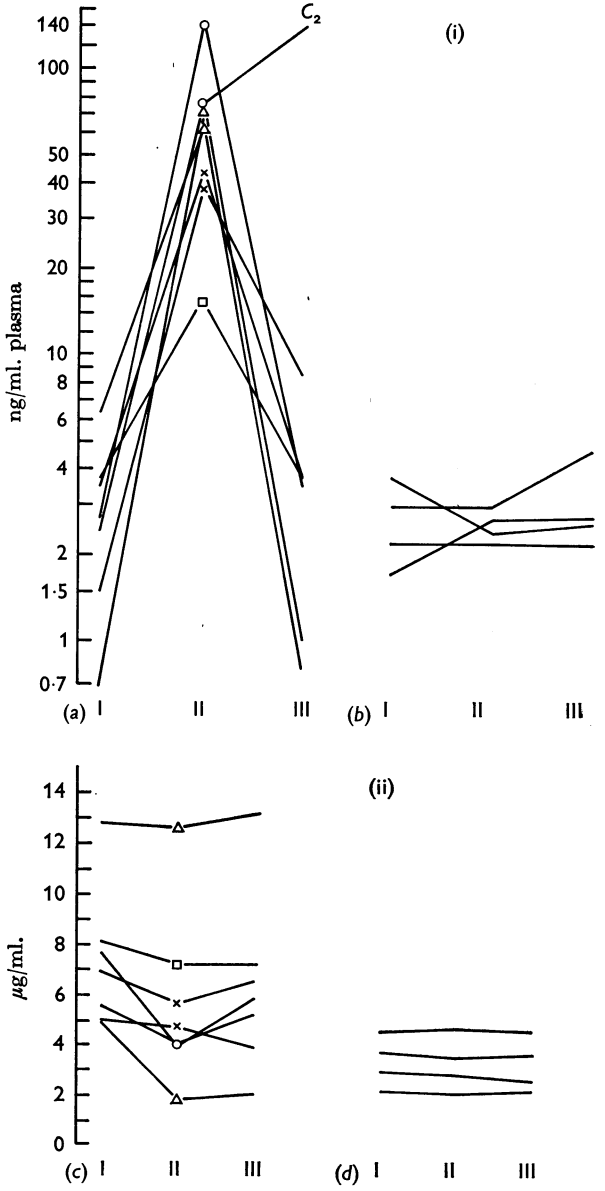

Fig. 2. (i) Free kinin assayed as bradykinin after concentration and purification. (*a*) Kinin levels in the plasma of four patients subject to the dumping syndrome. Three patients (denoted ○, × and △) were examined on two occasions: I, before experiment; II, at height of symptoms; III, 15–20 min after II, when symptoms had subsided in all cases except C_2. (*b*) Corresponding results from experiments with four normal volunteers. (ii) Kininogen in the samples as (i) assayed as bradykinin after activation by trypsin: (*c*) dumping subjects; (*d*) normal subjects.

DISCUSSION

Plasma contains a large amount of the inactive precursor α_2-globulin from which kinins may be released enzymically by kallikreins carried in the blood and in other body fluids (Schachter, 1964). Once released, the kinins are very rapidly destroyed by peptidases present in most body tissues (Amundsen & Nustad, 1965), including blood. The presence of these enzyme systems throughout the body, and the important pharmacological effects produced by minute amounts of kinins, suggest their involvement as mediators in a variety of physiological and pathophysiological processes. These include regulation of blood flow, and the mediation of local and general responses of the body to stress and injury, such as inflammatory and allergic reactions, and shock.

There is as yet little undisputed evidence for the release of free plasma kinin in such processes, and there have been few clinical studies, presumably because of the methodological problems involved. The half-life of synthetic bradykinin in the body is less than 0·5 min (Saameli & Eskes, 1962; McCarthy, Potter & Nicolaides, 1965), and kinin activity is easily lost during purification. The inadvertent production of kinins during sampling and the separation of plasma is an even greater hazard.

The methods previously described for the estimation of plasma kinin and kininogen have required separate samples for the estimation of each substance and immediate processing of the samples, thus being impracticable in most clinical situations. In addition, most methods for free kinin assay either require large volumes of blood, give low recovery, or are relatively unspecific.

The method now described enables both free kinin and its precursor to be estimated in the same 6 ml. sample of peripheral venous blood. It also includes haematocrit estimation which affords a check on possible changes due to fluid shift. The procedure separates free kinin from interfering potassium and biological amines, and gives the same relatively high recovery of added kinin over the range 10–50 ng/ml. of blood. The sample may be stored for about 8 hr after a very simple inactivation procedure, before further processing. Kinins are soluble in 80–90 % ethanol (Rocha e Silva, Beraldo & Rosenfeld, 1949) at which concentration proteins and enzymes are precipitated and temporarily inactivated. Stabilization and extraction by ethanol have been used in plasma kinin estimations (e.g. Allwood & Lewis, 1964; Brocklehurst & Lahiri, 1962). The kinin may be further purified and concentrated by a butanol extraction procedure adapted from that first used for the purification of 'hypertensin' (Clark, Winkler, Gollan & Fox, 1954) and later used for a direct extraction of kinin from urine (Gomes, 1955; Gaddum & Horton, 1959). This permits

bio-assay of much lower levels of kinin in the blood sample and largely eliminates most of the substances likely to interfere with bio-assay when such low levels have to be estimated. The amount of 5-HT added to samples in the control experiments was very large in order to match the levels found in blood during carcinoid flush. Although more than 95% was removed by the extraction process, the 5-HT inhibitor bromolysergic acid diethylamide (2-brom lysergide) was used to exclude the possibility of interference during bio-assay. The method of incubation of the alcohol-denatured whole blood proteins with trypsin to release the potential kinin from its precursor is similar to that used for plasma by Brocklehurst & Lahiri (1962) and Diniz & Carvalho (1963).

The method has been used to examine plasma kinin and kininogen levels in several groups of 'normal' individuals, and in conditions characterized by flushing and hypotensive episodes. The low normal level of free plasma kinin equivalent to 2·8 ng bradykinin/ml. plasma seems to be a very constant factor. Even this low value is artificially raised, since when samples contained no detectable activity, the value used for calculation was taken as the bio-assay threshold. In addition, it is possible that the trauma of sample-taking may cause some kinin release. The low levels in the bloodstream may simply reflect a much higher concentration in tissues or organs where kinins have a physiological role.

The results obtained with carcinoid patients support those previously obtained by Oates, Melman, Sjoerdsma, Gillespie & Mason (1964). These workers, using a method involving ethanolic inactivation of blood enzymes, followed by an ion exchange purification, found increased levels of kinin in hepatic venous blood during the carcinoid flush. It has been suggested (Webster & Gilmore, 1965) that the raised free kinin levels reported by others may have been the result of the activation of kinin release during the sampling procedure. The low 'normal' levels of free plasma kinin both in healthy volunteers and in patients in the absence of symptoms, and the consistent finding of raised levels associated with the onset of vasomotor symptoms, indicate that activation of kinin release is directly related to the symptoms and is not a methodological artifact. Levels greater than 10 ng/ml. plasma were invariably associated with vasomotor symptoms both in the carcinoid and dumping patients and in the two 'normal' subjects who suffered vasovagal fainting while the samples were being taken.

Measurements of the kininogen level on a single occasion have little value, since we have seen no obvious relation between levels above or below the mean 'normal' level of 6·1 μg Bk.eq./ml. plasma and any pathological or other conditions. However, in the subjects undergoing carcinoid and dumping syndromes studied by Zeitlin & Smith (1966) the sudden

release of a large amount of free kinin has always been accompanied by a simultaneous fall in the kininogen level.

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REFERENCES

ALLWOOD, M. J. & LEWIS, G. P. (1964). Bradykinin and forearm blood flow. *J. Physiol.* **170**, 571–581.

AMUNDSEN, E. & NUSTAD, K. (1965). Kinin-forming and destroying activities of cell homogenates. *J. Physiol.* **179**, 479–488.

BROCKLEHURST, W. E. & LAHIRI, S. C. (1962). The production of bradykinin in anaphylaxis. *J. Physiol.* **160**, 15–16*P*.

CIRSTEA, M., SUHACIU, G. & BUTCULESCU, I. (1966). Evaluation du rôle de la bradykinine dans le choc anaphylactique. *Archs int. Pharmacodyn. Ther* **159**, 18–33.

CLARK, L. C., WINKLER, C., GOLLAN, F. & FOX, R. P. (1954). The extraction and purification of hypertensin (Angiotonin). *J. biol. Chem.* **206**, 717–724.

DINIZ, C. R. & CARVALHO, I. F. (1963). A micromethod for determination of bradykininogen under several conditions. *Ann. N. Y. Acad. Sci.* **104**, 77–89.

EDERY, H. & LEWIS, G. P. (1963). Kinin-forming activity and histamine in lymph after tissue injury. *J. Physiol.* **169**, 568–583.

EISEN, V. & KEELE, C. A. (1966). Possible modes of kinin formation in some pathological states in man. In *Hypotensive Polypeptides*, pp. 551–564. Berlin: Springer.

GADDUM, J. H. & HORTON, E. W. (1959). The extraction of human urinary kinin (substance Z) and its relation to the plasma kinins. *Br. J. Pharmac. chemother.* **14**, 117–124.

GADDUM, J. H., PEART, W. S. & VOGT, M. (1949). The estimation of adrenaline and allied substances in blood. *J. Physiol.* **108**, 467–481.

GOLDFINGER, S., MELMAN, K. L., WEBSTER, M. E., SJOERDSMA, A. & SEEGMILLER, J. E. (1964). The presence of a kinin-peptide in inflammatory synovical effusions. *Arthritis Rheum.* **7**, 311–312.

GOMES, F. (1955). A slow contracting substance in normal human urine. *Br. J. Pharmac. chemother.* **10**, 200–207.

GOODWIN, L. G. & BOREHAM, P. F. L. (1966). Pharmacologically active peptides in trypanosome infections. In *Hypotensive Polypeptides*, pp. 545–549. Berlin: Springer.

GOODWIN, L. G., JONES, C. R., RICHARDS, W. H. G. & KOHN, J. (1963). Pharmacologically active substances in the urine of burned patients. *Br. J. exp. Path.* **44**, 551–560.

GREEF, K., SCHARNAGEL, K., LÜHR, R. & STROBACH, H. (1966). Die Abnahme des Kininogengehaltes des Plasmas beim toxischen, anaphylaktischen und anaphylaktoiden Schock. *Arch. exp. Path. Pharmak.* **253**, 235–245.

MARTINEZ, A. R., CARVALHO, I. F. & DINIZ, C. R. (1962). The bradykininogen content of plasma from women in labor. *J. Obstet. Gynaec. Br. Commonw.* **69**, 1011–1014.

MCCARTHY, D. A., POTTER, D. E. & NICOLAIDES, E. D. (1965). An *in vivo* estimation of the potencies and half-lives of synthetic bradykinin and kallidin. *J. Pharmac. exp. Ther.* **148**, 117–122.

OATES, J. A., MELMAN, K., SJOERDSMA, A., GILLESPIE, L. & MASON, D. T. (1964). Release of a kinin peptide in the carcinoid syndrome. *Lancet* i, 514–517.

PERITI, P. & GASPARRI, F. (1966). Bradykinin in the blood of women during pregnancy, labour and puerperium. In *Hypotensive Polypeptides*, pp. 536–543. Berlin: Springer.

ROCHA E SILVA, M., BERALDO, W. T. & ROSENFELD, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulins by snake venoms and by trypsin. *Am. J. Physiol.* **156**, 261–273.

ROCHA E SILVA, M. & ROSENTHAL, S. R. (1961). Release of pharmacologically active substances from the rat skin *in vivo* following thermal injury. *J. Pharmac. exp. Ther.* **132**, 110–116.

SAAMELI, K. & ESKES, T. K. A. B. (1962). Bradykinin and cardiovascular system: estimation of half-life. *Am. J. Physiol.* **203**, 261–265.

SCHACHTER, M. (1964). Kinins—a group of active peptides. *A. Rev. Pharmac.* **4**, 281–292.

WEBSTER, M. E. & GILMORE, J. P. (1965). The estimation of the kallidins in blood and urine. *Biochem. Pharmac.* **14**, 1161–1164.

ZEITLIN, I. J. & SMITH, A. N. (1966). 5-Hydroxyindoles and kinins in the carcinoid and dumping syndromes. *Lancet* ii, 986–991.