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MEASUREMENT OF URINARY KALLIKREIN ACTIVITY BY KININ RADIOIMMUNOASSAY*

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Abstract—A method for measuring urinary kallikrein activity by kinin radioimmunoassay (RIA) is described. Kinins were generated by incubating urine with partially purified dog kininogen in the presence of peptidase inhibitors. Antibodies against kallidin were induced in rabbits by injecting kallidin coupled to ovalbumin. One of the antisera generated was used at a final dilution of 1:18,000 to obtain a 40 per cent binding of approximately 3000 cpm (10 pg) of bradykinin (8-tyrosin)-[^{125}I]triacetate ([^{125}I]bradykinin). Synthetic kallidin (10–500 pg) was used to construct standard curves. When kinins generated by urinary kallikrein were also used, the two displacement curves for [^{125}I]bradykinin were similar. The RIA was sensitive to 10 pg kinins. The antiserum cross-reacted with bradykinin, methionyl-kallidin, and kininogen, but not with angiotensin, oxytocin or SQ 20,881. To increase the specificity of the RIA, the kininogen was removed by ethanol precipitation followed by QAE-Sephadex-A50 chromatography. Kallikrein activity in 81 human urine samples and 8 samples obtained from a dog undergoing stop-flow procedure was measured by RIA and bioassay. Correlations of $r = 0.81$ and 0.94 were found. This RIA is useful for measuring kallikrein activity in rat, dog and human urine.

The physiological significance of urinary kallikrein is not known. However, it has been reported that excretion of this enzyme is decreased in patients with essential hypertension [1, 2] and in experimental animals with renal hypertension [3]. It has also been implicated in the regulation of blood pressure [4] and sodium balance [5, 6].

Kallikrein is usually measured by its esterolytic activity over synthetic ester substrates or by its capacity to generate kinins (kininogenic activity) [7]. Because there are urinary esterases that have no kininogenic activity [8, 9], methods using the esterolytic properties of kallikrein are less specific than kininogenic methods. Unfortunately, in methods based on kininogenic activity the kinins generated are measured by a variety of bioassays which also have low specificity [7]. A radioimmunoassay (RIA) for determining kinins generated would enable kallikrein activity to be measured with greater reliability and, in turn, facilitate investigation of the kallikrein–kinin system in physiological or pathological situations.

RIA procedures have been described [10–12] for determination of blood kinin levels; however, these methods have not been applied to measurement of kallikrein activity. This report describes a method for measuring urinary kallikrein activity using a RIA. The RIA is specific for kinins and detects a minimum of 10 pg kallidin.

In addition, problems encountered with kininogen interference are discussed.

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MATERIALS AND METHODS

Antibody. Kallidin was coupled to ovalbumin using carbodiimide as described by Goodfriend *et al.* [13]. Coupled antigen (1 mg) was emulsified in complete Freund's adjuvant and injected into the paws of three New Zealand rabbits once weekly for 3 weeks; thereafter, booster injections in incomplete Freund's adjuvant were given every month. Antisera were collected 7–10 days after the booster injection. The antisera were heated to 60° for 30 min and stored frozen.

Antigen. Bradykinin (8-tyrosin)-[^{125}I]triacetate ([^{125}I]bradykinin) was purchased from New England Nuclear (Boston, Mass.), and more recently from Nuclear International Corp. (Burlington, Mass.). It was also generously supplied by Dr. T. Goodfriend (Madison, Wisc). Each batch of commercial antigen was purified by adsorption with Amberlite IRC-50 resin according to the procedure of Talamo *et al.* [10]. Synthetic bradykinin, kallidin, methionyl-kallidin and angiotensin I were purchased from Schwarz/Mann (Orangeburg, N.Y.), SQ 20,881 was obtained from Squibb Inst. (Princeton, N.J.) and oxytocin from Parke, Davis & Co. (Detroit, Mich.).

Dextran-coated charcoal. Dextran-coated charcoal was prepared by a modification of the method of Herbert *et al.* [14] as follows: 6.25 g Norit "A" charcoal (Amend Drug & Chemical Co., Irvington, N.J.) was dissolved in 125 ml of barbital buffer, pH 7.4; 6.25 g Dextran T70 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was also dissolved in 125 ml of the barbital buffer. The charcoal suspension and Dextran solution were combined and mixed thoroughly and the volume was brought to 1000 ml with barbital buffer.

Standard plot. The RIA was performed in 0.1 M Tris–HCl buffer, pH 7.4, containing 0.2% gelatin and 0.1% Neomycin (Tris buffer). The incubation mixture

contained 0.1 ml [^{125}I]bradykinin (approximately 3000 cpm), 0.1 ml of 1:3000 dilution of antiserum, kallidin standard ranging from 10 to 500 pg or 2 to 20 μl of the unknown sample, and Tris buffer to obtain a final volume of 0.6 ml. Samples were incubated for 24 hr at 4°; 6 mg of Dextran-coated charcoal was used to separate free kinins from antibody-bound kinins. The supernatant was decanted, and free [^{125}I]bradykinin in the charcoal was counted in an automatic gamma spectrometer (Packard). The standard curve was obtained by plotting the per cent of initial binding (B/B_0) against the kallidin standards. B and B_0 were calculated by subtracting charcoal-adsorbed counts (free) from the corrected total count. Total counts were corrected for unspecific binding to the gelatin Tris buffer. Duplicate estimations were made for each point on the curve, and the unknown samples were extrapolated from this plot or calculated by computer using logit transformation as described by Rodbard *et al.* [15].

The RIA was performed at pH 7.0, 7.4 and 8.0. Binding of radiolabeled antigen to the antibody was also studied by varying incubation time from 4 to 24 hr.

The specificity of the RIA was evaluated by using bradykinin, kallidin, methionyl-kallidin, purified dog kininogen, angiotensin I, oxytocin and SQ 20,881.

Generation and isolation of kinins. Kinins were generated by incubating urine with kininogen in the presence of kininase inhibitors. The kininogen was partially purified from dog plasma by ammonium sulfate precipitation [16] or DEAE chromatography as suggested by Guimaraes *et al.* [17]. The substrate concentration was expressed as the amount of kinins formed when incubated with an excess of trypsin. The reaction of urinary kallikrein and kininogen was carried out in 0.1 M phosphate buffer, pH 8.5, containing 0.2% bacitracin, 3.0 mM 1,10-phenanthroline, and 30 mM Na_2 ethylenediamine tetraacetic acid. Varying amounts of urine (2–100 μl) were brought to a final volume of 0.5 ml with phosphate buffer and warmed at 37° for 5 min. Two thousand ng of prewarmed kininogen (37°) in 0.5 ml phosphate buffer was added to each sample to initiate the reaction, and the samples were incubated for 15 min at 37°. The enzymatic reaction was terminated by adding 4 ml of 96% ethanol, then allowed to stand for 5 min at room temperature and centrifuged at 1283 g for 10 min at 4°. The precipitates were washed with 1 ml of 80% ethanol and the combined supernatants evaporated under a dry stream of nitrogen to a residual volume of 0.5 ml. The samples were then passed through QAE-Sephadex-A50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) columns, 0.5 × 2 cm.* The gel was equilibrated and eluted with 0.0075 M Tris–HCl buffer, pH 8.0, containing 0.2% bacitracin. The effluent and the two washings of 2.0 ml each were combined and brought to a final volume of 5.0 ml. Aliquots of 2–20 μl were tested in RIA to determine kinin concentration.

Prior to standardization of the above procedure, kinins were isolated by precipitation with trichloroacetic acid (TCA) (8.5%) followed by Amberlite IRC-50 chromatography, essentially as described by Talamo *et al.* [10]. The individual steps outlined above were evaluated for recovery of [^{125}I]bradykinin, kallidin and kininogen. In addition, a standard of crude kallikrein was prepared by precipitating the proteins from a pool of rat urine with 80 per cent saturation of ammonium sulfate. The kinin-generating capacity of crude kallikrein standard was estimated in each set of experiments and used as a reference preparation.

The effects of incubation time, substrate concentration and amount of urine in the generation of kinins were also studied. Siliconized glassware was used in all steps of the procedure, since kinins at low concentration are rapidly adsorbed to glass.

Determination of kallikrein activity in human and dog urine by bioassay and RIA. Urine was collected from 81 individuals during a 24-hr period. Eight urine samples were also collected from a dog undergoing a stop-flow procedure. Kallikrein activity was estimated by the bioassay technique of Marin-Grez and Carretero [18] and by RIA.

RESULTS

High-titer antibody was produced by one of the rabbits. It was possible to use this antiserum in a final dilution of 1:18,000 to obtain 35–45 per cent binding of approximately 3000 cpm (10 pg) [^{125}I]bradykinin. Maximum binding of [^{125}I]bradykinin was obtained after 20 hr of incubation at 4° (Fig. 1). Figure 2 shows a typical standard curve for synthetic kallidin. It also shows displacement of [^{125}I]bradykinin by kinins generated by urinary kallikrein. The influence of pH on the standard curve is shown in Fig. 3. The cross-reactivity of antikallidin serum with bradykinin, kallidin, methionyl-kallidin, kininogen, angiotensin I, oxytocin and SQ 20,881 is shown in Table 1.

Table 2 gives the values obtained by direct measurement of kininogen by RIA for kinins before and after its removal by the procedures described. Kininogen values are expressed as a per cent of their total capacity to release kinins when incubated with an excess of trypsin. Note that results in Table 2 were obtained when kininogen was added directly to the RIA without prior incubation with trypsin. This is

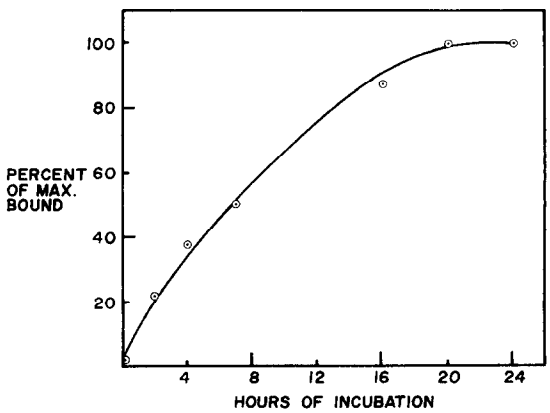

Fig. 1. Percentage of binding of [^{125}I]bradykinin at different times of incubation (4°). Maximum bound (100 per cent) = binding obtained at 24 hr.

*J. Spragg, personal communication.

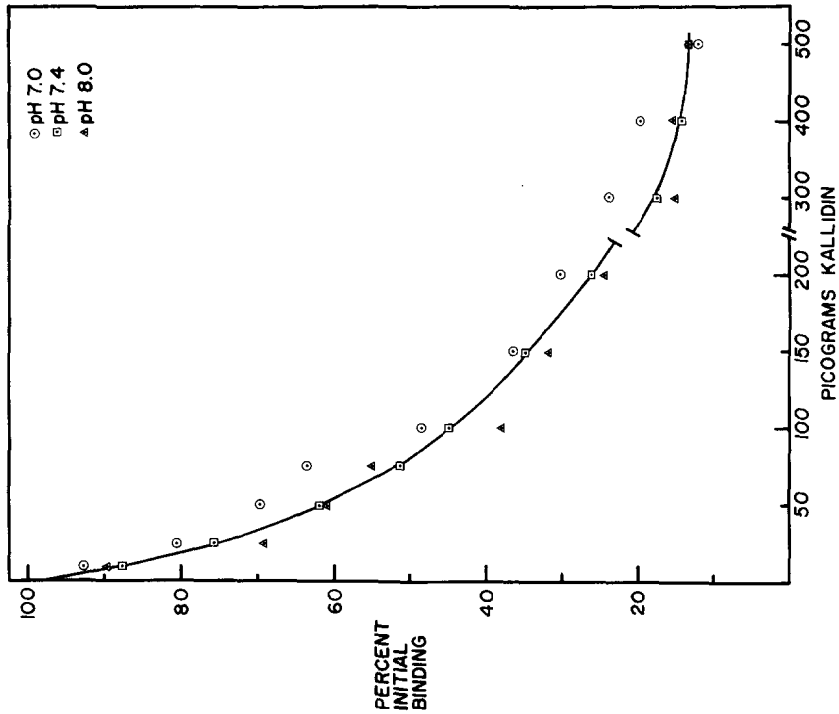

Fig. 3. Influence of different pH values on the radioimmunoassay.

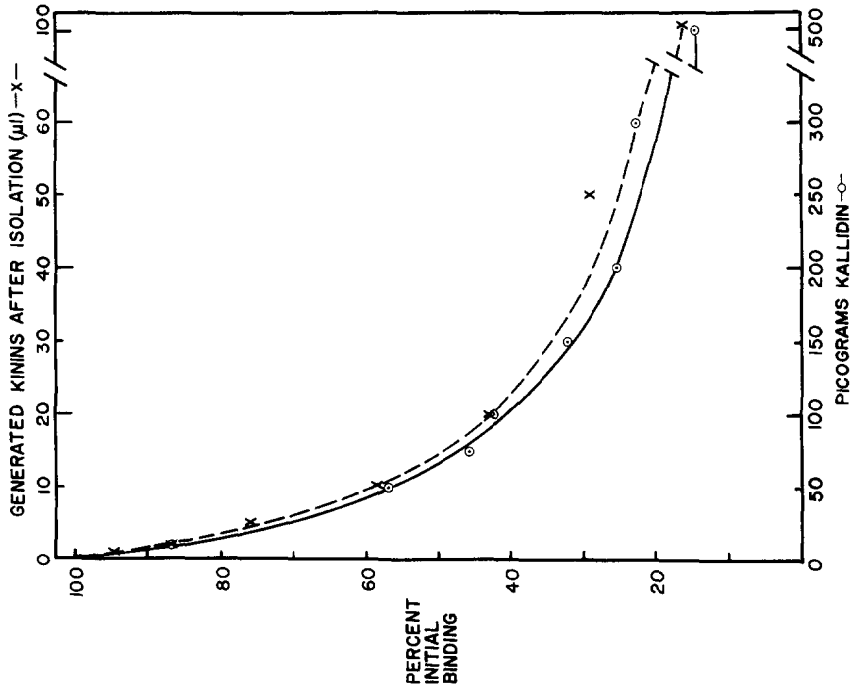

Fig. 2. Standard curves with unlabeled synthetic kallidin (circles with unbroken line) and with kinins generated by urinary kallikrein and subsequent isolation by ethanol precipitation and QAE-Sephadex as described in the text (× with broken line).

Table 1. Relative capacity, by unit of weight, of different peptides to displace [^{125}I]bradykinin from the antibody*

Kallidin	Brady-kinin	Met-Lys Brad	Kinino-gen	Ang I	Oxy-tocin	SQ 20.881
100	153	83	84	< 0.001	< 0.001	< 0.001

* Kallidin (lysyl-bradykinin) is arbitrarily taken as 100.

Table 2. Kininogen blank before and after its removal by various procedures*

Untreated kininogen	8.5% TCA	IRC-50	TCA + IRC-50	Ethanol + IRC-50	Ethanol + QAE-Sephadex
91.0	7.8	21.5	5.5	2.8	0–2

* Kininogen is expressed as a per cent of the amount originally added to the assay. For details, see text.

Table 3. Per cent of recovery of [^{125}I]bradykinin and kallidin by three different methods of separation

Method of separation	Kinin	
	[^{125}I]bradykinin	Kallidin
TCA + IRC-50	74–88	
Ethanol + IRC-50		76–84
Ethanol + QAE-Sephadex	84–96	86–96

referred to as the kininogen blank. It should be pointed out that untreated kininogen reacted with the antibody almost identically to synthetic kallidin.

Table 3 shows recovery of kinins after different procedures of isolation.

The relationship between kinin generation and time of incubation was linear (Fig. 4). Kinin formation increased in proportion to the amount of urine used (Fig. 5). When 2–10 μl of rat urine or 100 μl of human urine was incubated without kininogen (urine blank), no kinins were detected. When 5 μl of rat urine was incubated with varying amounts of kininogen, the reaction velocity showed a hyperbolic dependence on substrate concentration (Fig. 6). Less than 10 per cent of the substrate was consumed in the assays; reactions

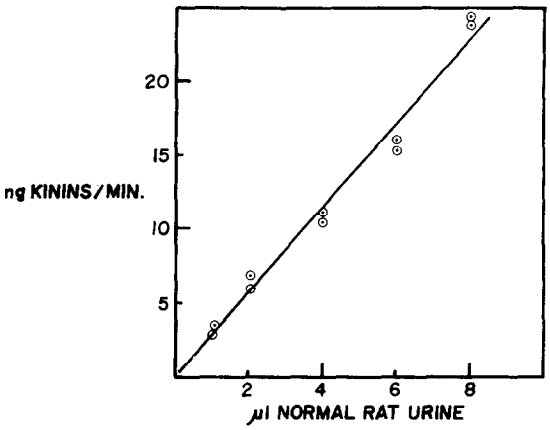

Fig. 5. Enzyme concentration: different volumes of rat urine were incubated with 2000 ng substrate for 15 min.

consuming a higher amount were repeated using less urine.

Table 4 shows results obtained when 0.1 mg of kallikrein standard was incubated with substrate in sextuplicate on day 1, and in quadruplicate on days 2 and 3. The intra-assay coefficient of variation was 4.4 per cent, while the interassay was 6.5 per cent. After

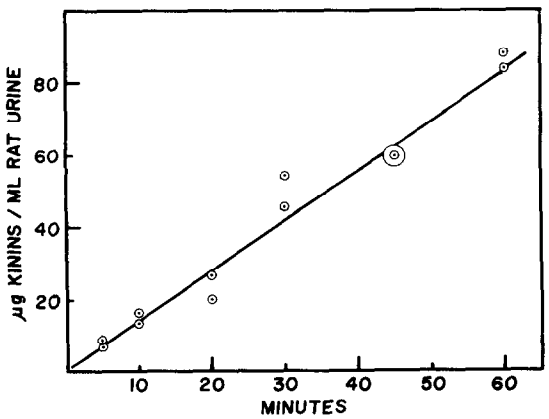

Fig. 4. Kinin formation at different incubation times: 2 μl of rat urine was incubated with 2000 ng substrate.

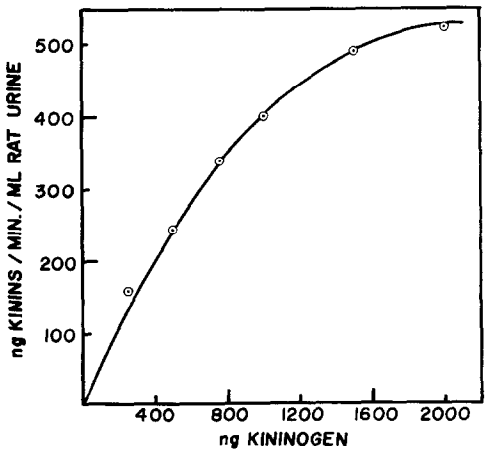

Fig. 6. Substrate concentration: different amounts of substrate were incubated with 2 μl urine for 15 min.

Table 4. Results obtained with RIA when identical kallikrein standards (0.1 mg) were incubated with substrate*

	Kallikrein standard (0.1 mg)							
Day	1	2	3	4	5	6	$\bar{X}$	S.D.
1	1.58	1.51	1.54	1.56	1.60	1.46	1.54	0.05
2	1.53	1.62	1.58	1.63			1.63	0.05
3	1.53	1.50	1.37	1.33			1.43	0.10
$\bar{X}$							1.53	0.10

* On day 1 the samples were incubated in sextuplicate; on days 2 and 3, in quadruplicate. Numbers in the table indicate ng kinins generated/μg of protein/min of incubation. $\bar{X}$ = mean, S.D. = standard deviation.

enzyme substrate incubation, samples were tested in duplicate by RIA, and the coefficient of variation of the RIA itself was 2.94 per cent.

Kallikrein activity, measured by bioassay and RIA in 81 samples of human urine, is shown in Fig. 7. A good correlation ($r = 0.86$) was obtained ($P < 0.001$). In eight dog urine samples, a correlation of 0.94 ($P < 0.001$) was obtained.

DISCUSSION

Although RIA has been used for determination of circulating blood kinin levels [10, 12], it has not been used for measurement of kallikrein activity. This report describes a method for measurement of kallikrein activity in urine by a kinin RIA. For this, urinary kallikrein was incubated with plasma kininogen; the kinins released were subsequently measured by RIA. The validity of this assay was verified by the following: amounts of kinins generated were proportional to the amount of urine (enzyme) used when the substrate was kept constant (Fig. 5); when the same samples were tested repeatedly (Table 4), results were consistent; and a highly significant correlation was found between urinary kallikrein activity measured by RIA and by bioassay (Fig. 7).

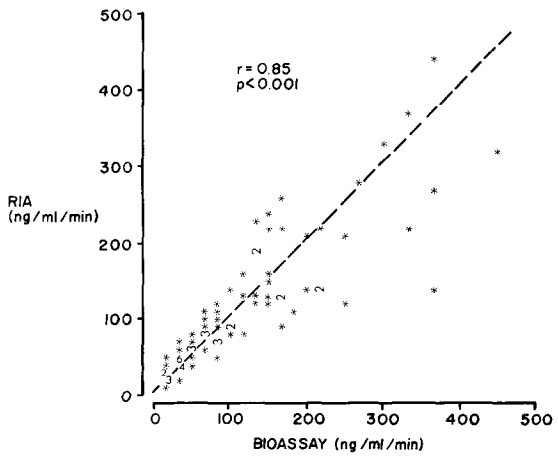

Fig. 7. Comparison of kallikrein activity in 81 human urine samples, as determined by bioassay and by radioimmunoassay. Asterisks indicate individual values; numerals represent the number of urine samples with identical results.

Since urinary kallikrein releases kallidin, the antibody used in this RIA was generated with kallidin as antigen. This antibody is the first generated against kallidin and has one of the highest titers reported so far [10–12, 19]. However, the antibody cross-reacted with bradykinin, methionyl-kallidin and kininogen. Similar results have been reported with antibodies against bradykinin [10, 20]. Interference of substrate in the kinin RIA has not been investigated previously. It was found that the kininogen blanks were still high (Table 2) even though TCA precipitation and IRC chromatography eliminated a considerable amount of kininogen. This could be due to the following reasons: first, the isolation procedure could release kinins from the substrate; second, a small amount of kininogen could still be present in the samples; or third, a combination of both could be the case. We found that kininogen blanks were much lower when ethanol precipitation and QAE-Sephadex chromatography were combined. For this reason, this procedure was finally adopted.

Kininogen blanks were run in our kallikrein assay and, if detectable activity was more than 5 ng kinin/2000 ng of kininogen, the kininogen was rejected. Furthermore, if the kininogen blank was more than 10 per cent of the kinin generated by urinary kallikrein, the sample was rerun with higher amounts of urine. However, since the reaction was not zero-order (Fig. 6), care was taken not to consume more than 10 per cent of the substrate. This was done to avoid a change in initial velocity of the reaction due to excessive substrate consumption. The urine blanks in the small amounts used for the incubation were consistently negative, indicating that there was no interference by the urinary kinins [21].

Precautions should be taken to avoid kininogen interference with the kinin assay. This could be done either by totally removing the kininogen or by using an antibody that does not cross-react with kininogen. Other investigators [10–12] have not reported whether their antibodies against bradykinin cross-reacted with kininogen. It has been reported that antibodies against kininogen cross-react with kinins [22], but it cannot be concluded from this that the inverse will always be the case. Cross-reaction of antibodies against kinins with kininogen becomes very important when the antibody is used to measure circulating kinins, especially if one takes into consideration that reported levels of kinins/ml of blood or plasma vary from 0 to 5 ng [10, 12, 23], and kininogen levels vary between 5 and 10 μg [24]. It becomes apparent that, when kinins are being measured, slight contamination by kininogen will be sufficient to give a deceptively high level of kinin. This would be the case, at least, with our antibody, which cross-reacts with kininogen.

Finally, the method reported here, due to its specificity and sensitivity, could contribute to the understanding of the physiological and pathological role of renal kallikrein. In addition, this assay could also be easily modified to measure kallikrein activity in other body fluids and tissues.

REFERENCES

1. A. H. Elliot and F. R. Nuzum, *Endocrinology* **18**, 462 (1934).

2. H. S. Margolius, R. Geller, J. J. Pisano and A. Sjoerdsma, *Lancet* **II**, 1963 (1971).
3. H. R. Croxatto and M. San Martin, *Experientia* **26**, 1216 (1970).
4. H. S. Margolius, R. G. Geller, W. DeJong, J. J. Pisano and A. Sjoerdsma, *Circulation Res.* **30–31** (Suppl. II), 125 (1972).
5. A. Adetuyibi and I. H. Mills, *Lancet* **III**, 303 (1972).
6. O. A. Carretero and N. B. Oza, in *Mechanisms of Hypertension* (Ed. M.P. Sambhi), p. 290. Excerpta Medica, Amsterdam (1974).
7. I. Trautschold, in *Handbook of Experimental Pharmacology* (Ed. E. Erdös), Vol. 25, p. 52. Springer, New New York (1970).
8. V. Hial, C. R. Diniz and M. Mares-Guia, *Biochemistry* **13**, 4311 (1974).
9. K. Nustad and J. V. Pierce, *Biochemistry* **13**, 2312 (1974).
10. R. C. Talamo, E. Haber and K. F. Austen, *J. Lab. clin. Med.* **74**, 816 (1969).
11. T. L. Goodfriend and D. L. Ball, *J. Lab. clin. Med.* **73**, 501 (1969).
12. M. L. Mashford and M. L. Roberts, *Biochem. Pharmac.* **21**, 2727 (1972).
13. T. L. Goodfriend, L. Levine and G. D. Fasman, *Science, N.Y.* **144**, 1344 (1964).
14. V. Herbert, K. S. Lau, C. W. Gottlieb and S. J. Bleicher, *J. clin. Endocr. Metab.* **25**, 1375 (1965).
15. D. Rodbard, P. L. Rayford, J. A. Cooper and G. T. Ross, *J. clin. Endocr. Metab.* **28**, 1412 (1968).
16. M. Marin-Grez, N. B. Oza and O. A. Carretero, *Henry Ford Hosp. med. J.* **21**, 85 (1973).
17. J. A. Guimaraes, R. C. Lu, M. E. Webster and J. V. Pierce, *Fedn Proc.* **33**, 641 (1974).
18. M. Marin-Grez and O. A. Carretero, *J. appl. Physiol.* **32**, 428 (1971).
19. J. Spragg, K. F. Austen and E. Haber, *J. Immun.* **96**, 865 (1966).
20. M. L. Mashford and M. L. Roberts, in *Advances in Experimental Medicine and Biology* (Eds. N. Back and F. Sicuteri), Vol. 21, p. 23. Plenum Press, New York (1972).
21. I. Miwa, E. G. Erdös and T. Seki, *Life Sci.* **7**, 1339 (1968).
22. J. Fischer, J. Spragg, R. C. Talamo, J. V. Pierce, K. Suzuki, K. F. Austen and E. Haber, *Biochemistry* **8**, 3750 (1969).
23. O. A. Carretero, A. Nasjletti and J. C. Fasciolo, *Experientia* **21**, 141 (1965).
24. O. A. Carretero, A. Nasjletti, A. Inon and R. S. Dohmen, *Am. J. med. Sci.* **259**, 182 (1970).