Biochemical Pharmacology, 1962, Vol. 11, pp. 707–714. Pergamon Press Ltd., Printed in Great Britain.

PARTIAL PURIFICATION OF THE PLASMA SUBSTRATE FOR THE BRADYKININ-RELEASING ENZYME FROM THE VENOM OF *BOTHROPS JARARACA*

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(*Received* 6 *January* 1962; *accepted* 30 *March* 1962)

Abstract—The substrate for the bradykinin-releasing enzyme from the venom of *Bothrops jararaca* was purified 170-fold from fresh horse plasma. Horton's substrate, prepared by acidification of plasma to pH 2·0, heating to 38 °C, and neutralization to pH 7·0 at low temperature, was fractionally precipitated with ammonium sulfate, and the fraction obtained between 0·33 and 0·50 saturation was chromatographed on diethylaminoethyl (DEAE)-cellulose. The activity of the bradykinin-releasing enzyme of the venom on crude plasma and on the fractions obtained during the course of purification of bradykininogen is compared with that of trypsin.

RELATIVELY crude mixtures of plasma proteins have been used as substrate for the plasma kinin-forming enzymes.[1] The substrate for the bradykinin-releasing enzyme from the venom of *Bothrops jararaca* was first found in the fraction that precipitates at 0·30–0·45 saturation with ammonium sulfate.[2] This fraction has been extensively used in the determination of enzymes that liberate bradykinin, in spite of the fact that it is strongly contaminated with plasma kinin-forming activity and kininase, the peptidase that destroys bradykinin. Werle's substrate,[3] which consists of plasma heated for 3 hr at 56 °C, has the advantage of being free from kinin-forming activity and kininase, but in this laboratory it was found to lose a great part of its bradykininogen, when it was obtained from horse plasma. Cohn's fraction IV-4, which was used as substrate for the release of bradykinin by Van Arman,[4] contains kininase. Horton[5] recently proposed a substrate that is prepared by acidifying the plasma to pH 2·0, heating it at 37 °C for 15 min and neutralizing to pH 7·0. Working with dog plasma he obtained a preparation free from kininase and practically free from kallikrein, one of the kinin-forming enzymes from plasma. We were unable, with horse plasma to obtain a preparation free from kinin-forming activity; on the contrary, this was enhanced in the substrate preparation. Most of the preparations mentioned above are very crude mixtures of proteins and, apart from being contaminated by kininase and kinin-forming enzymes, may contain inhibitors of these enzymes.

This paper describes the attempts we made to obtain a purer substrate for the bradykinin-releaser of the venom of *Bothrops jararaca*, free from these highly undesirable contaminants.

* Supported in part by Grant RF 58217 from the Rockefeller Foundation.
† Supported in part by the Instituto Butantan Research Funds.

METHODS

Bradykinin-releasing enzyme. A 2 per cent solution of *Bothrops jararaca* venom in saline (0·9% NaCl) was heated in a water bath to 87 °C, immediately cooled in an ice bath, centrifuged, and the supernatant fractionated with ammonium sulfate as previously described;[6] the fraction precipitated at 0·60–0·70 saturation was dialyzed against saline until free from NH_4^+ ions. This preparation (P53) hydrolyzed 6·6 μmole *p*-toluene sulfonyl-L-arginine methyl ester (TAMe)/min/mg protein compared with crystalline trypsin which hydrolyzed 82 μmole TAMe/min/mg protein.

Estimation of bradykinin. The assay of bradykinin was performed on the isolated guinea-pig ileum. Pieces of terminal ileum removed from animals weighing 180–250 g were suspended in a 7 ml-muscle chamber containing aerated Tyrode's solution with atropine (1 μg/ml, as base) and diphenhydramine (1 μg/ml, as base) and maintained at 35 °C. Longitudinal contractions were recorded with a frontal lever with a load of 1–1·5 g, giving a sixfold magnification.

Bradykininogen assay. This was accomplished by the direct method in which the bradykinin-releasing enzyme was added to the bath 1 min after the sample of substrate to be tested, and the release of bradykinin was observed during 3 min, or by the method of Diniz and co-workers[7], slightly modified as follows. The samples were incubated with 200 μg of crystalline trypsin or 200 μg of venom enzyme expressed as protein. Previous assay with plasma using 50, 100, 200, and 400 μg of any of the enzymes, expressed as protein, gave the same release of bradykinin for each enzyme, showing that an excess was already present. Immediately after the incubation at 37 °C the samples were frozen and kept in the cold until tested for bradykinin. The method of Diniz *et al.* has the advantage that the kinin-releasing activity and kininase that might be present in the substrate are inactivated prior to the incubation with the bradykinin-releasing enzyme. Comparing samples of plasmas of ten different horses we obtained the same bradykininogen level, whether we utilized either this procedure or the complete method of Diniz *et al.*[7] When using trypsin as a bradykinin-releasing enzyme, the levels of horse-plasma bradykininogen observed were within the range obtained by these authors for mammalian plasmas.[7]

When either the direct method or the method of Diniz *et al.* was used the relative potency of samples was calculated from the response to single and double doses[8] of bradykinin and substrate, expressed as protein.

Preparation of substrate. Horse blood was used as source of substrate. Blood coagulation was prevented by the addition of potassium oxalate in the concentration of 3 g per l of blood. Immediately after collection the blood was centrifuged in the cold, at 0–5 °C. The plasma was separated and acid-treated as recommended by Horton.[5] The plasma was adjusted to pH 2·0 with 1 M HCl, heated at 38 °C for 20 min, cooled in an ice bath and adjusted to pH 7·0 with 1 M $NaHCO_3$. Excessive foaming was prevented by the addition of small amounts of ether. The mixture was centrifuged in the cold, at 0–5 °C, and immediately afterward a saturated solution of ammonium sulfate was added to give 0·20 saturation. This prevented the action on the substrate of the bradykinin-forming activity which had been activated by the acid treatment. This mixture was left overnight, stirred for 30 min, and the precipitate removed by centrifugation. To attain the next levels of saturation, the ammonium sulfate solution was added slowly, with continuous stirring, to the desired saturation. The precipitate was centrifuged, dissolved in a small volume of saline (0·9% NaCl),

and dialyzed in the cold in cellophane tubing against saline until the diffusate was free of NH_4^+ ions. The fraction precipitated between 0·33 and 0·50 saturation with ammonium sulfate was dialyzed against 0·005 M sodium phosphate buffer, pH 7·0 in the cold, and chromatographed on (DEAE)-cellulose as soon as possible, or frozen until the chromatography could be performed, since formation of bradykinin from the substrate continued, even at 1 to 4 °C.

Chromatography. DEAE-cellulose was prepared by the method of Peterson and Sober[9] and the conditions of chromatography were similar to the procedure described by Sober *et al.*[10] The protein solution, equilibrated against 0·005 M sodium phosphate buffer, pH 7·0, was centrifuged for 10 min at 5000 **g** in the cold, before application to a column of DEAE-cellulose (2 × 40 cm) which previously had been fully equilibrated against the buffer. The column was mounted on a fraction collector in the cold room (5 °C) and the protein (1·7 g) was applied in 40-ml solution. Elution was carried out at 80-cm water pressure and a flow rate of 90 ml/hr, fractions of 6 ml being collected. A gradient was established by introducing concentrated buffer, 0·05 M NaH_2PO_4–0·1 M NaCl and 0·1 M NaH_2PO_4–0·2 M NaCl, from a separatory funnel into a chamber containing 125 ml of 0·005 M sodium phosphate buffer, pH 7·0. A simultaneous increase in the concentration of sodium phosphate and sodium chloride was thus obtained. The elution of protein was followed by measurement of extinction at 280 mμ. In the fractions corresponding to the peaks, protein determinations were made by the method of Lowry *et al.*[11] The presence of the substrate in the various fractions was followed by the direct method on the ileum, using the bradykinin-releasing enzyme of the venom. The chromatographic procedure and the preparation of the solutions were conducted at 2–5 °C.

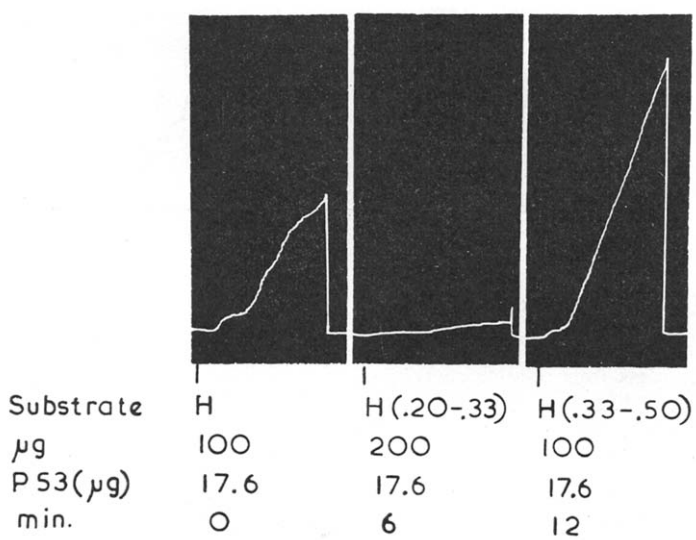

FIG. 1. Fractionation of Horton's substrate by ammonium sulfate. Assay of bradykininogen by the direct method. Isolated guinea-pig ileum suspended in a 7-ml bath containing aerated Tyrode's solution with atropine and diphenhydramine (1 μg/ml) at 35 °C. H = initial Horton's substrate; H (0·20–0·33) and H (0·33–0·50) = fractions respectively precipitated between 0·20 and 0·33, and 0·33 and 0·50 saturation with ammonium sulfate; P53 = partially purified bradykinin-releasing enzyme from the venom of *Bothrops jararaca*. The enzyme was added to the bath 1 min after the substrate, and the release of bradykinin was observed during 3 min. Doses μg protein.

Rechromatography. The most active fractions obtained during chromatography were combined and added to a 1·1 × 38 cm DEAE-cellulose column equilibrated with 0·05 M NaH_2PO_4 — 0·1 M NaCl. A gradient to 0·1 M NaH_2PO_4 — 0·2 M NaCl was applied as described.

RESULTS

Fig. 1 shows that after fractionation of Horton's substrate by ammonium sulfate, most of the active material was obtained in the fraction precipitated between 0·33 and 0·50 saturation with ammonium sulfate. After chromatography of this fraction on DEAE-cellulose (Fig. 2) several peaks of protein were obtained, the substrate of

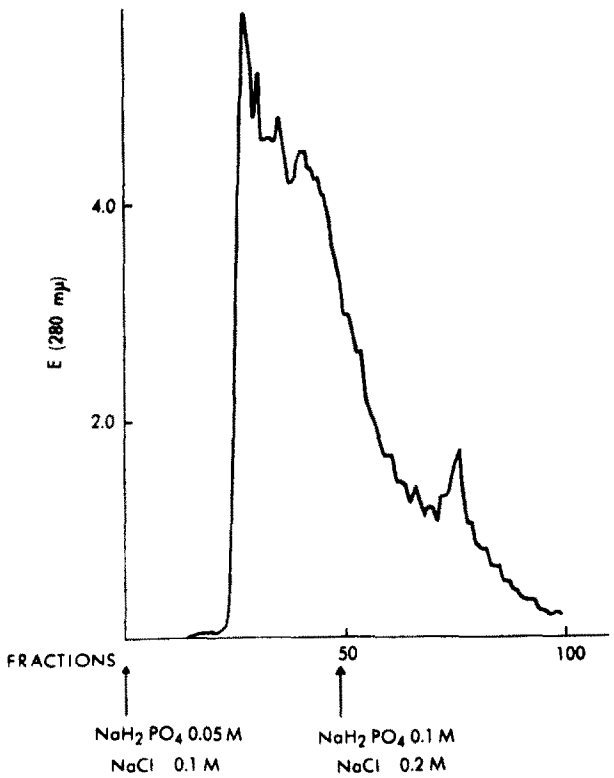

FIG. 2. Elution curve from solution containing the proteins precipitated from Horton's substrate between 0·33 and 0·50 saturation with ammonium sulfate, applied to a column of DEAE-cellulose. Gradient was made by running the buffers into 0·005 M sodium phosphate, pH 7·0.

bradykinin-releasing enzyme being eluted when the concentrations of NaH_2PO_4 and NaCl reached, respectively, the levels of 0·08 and 0·16 M (fractions 74 to 78) as shown in Fig. 3. These fractions had apparently been freed from kinin-forming enzyme, since no bradykinin could be detected prior to the addition of the enzyme from venom. The yield of protein, in the four most active fractions from the first chromatography, was 1·5 per cent, in the preparation described here, corresponding to 97·5 per cent of the substrate's initial activity. After rechromatography the yield of protein was reduced to 0·2 %, accounting for 34 % of the starting activity.

Table 1 shows the amount of bradykininogen per milligram of protein in the starting material (acid-treated crude plasma), in the fractions precipitated between 0·20 and 0·33 and 0·33 and 0·50 of saturation with ammonium sulfate, in the fraction of highest purity obtained after chromatography on a DEAE-cellulose column, and in a fraction obtained after rechromatography which corresponded to the peak of activity during the previous chromatography. It can be seen that the substrate for the bradykinin-releasing enzyme from the venom is concentrated in the fraction precipitated between 0·33 and 0·50 saturation with ammonium sulfate, and

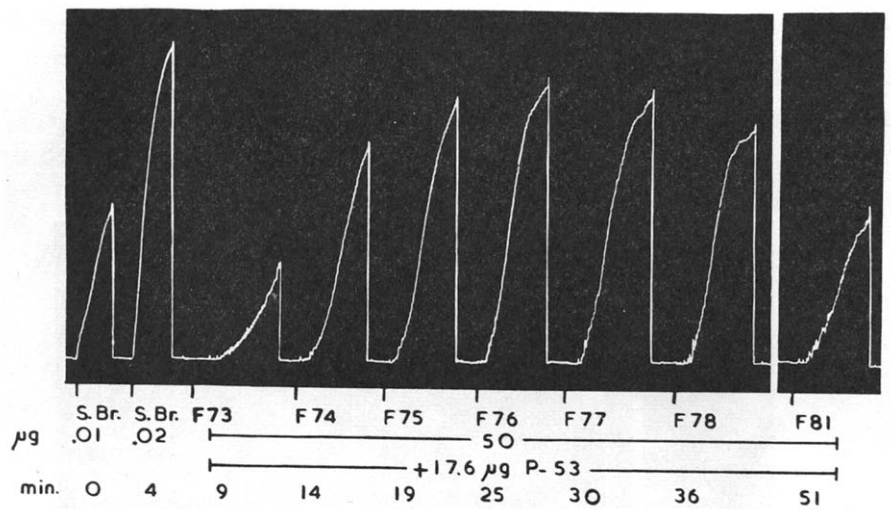

FIG. 3. Assay of bradykininogen, in terms of liberated bradykinin, on the isolated guinea-pig ileum (same conditions as in Fig. 1). S.Br. = synthetic bradykinin (μg); F74 to F81 = fractions obtained from the active material precipitated between 0·33 and 0·50 saturation with ammonium sulfate, by chromatography on DEAE-cellulose; P53 = venom enzyme. Doses of substrates and venom enzyme expressed in micrograms of protein. The bulk of activity was eluted in the fractions F74 to F78 free from bradykinin-releasing activity, since no contraction of the ileum was observed previous to the addition of the venom enzyme to the bath.

TABLE 1. BRADYKININOGEN CONTENT OF SAMPLES OBTAINED IN THE COURSE OF PURIFICATION OF THE HORSE PLASMA SUBSTRATE FOR THE BRADYKININ-RELEASING ENZYME FROM THE VENOM OF *Bothrops jararaca*

Fraction no.	Procedure	μg bradykinin released per mg substrate protein	
		Trypsin	Partly purified venom enzyme
1	Crude plasma, acid-treated	0·072	0·029
2	Precipitation at 0·20–0·33 saturation with $(NH_4)_2SO_4$	0·14	0·039
3	Precipitation at 0·33–0·50 saturation with $(NH_4)_2SO_4$	0·14	0·09
4	Chromatography on DEAE-cellulose (fraction 76)	1·11	1·9
5	Rechromatography of fractions 74–78 on DEAE-cellulose	3·14	5·0

Bradykininogen was assayed by the method of Diniz *et al.*[7] using trypsin or partly purified venom enzyme, in terms of liberated bradykinin.

that a purification of 170-fold was achieved by chromatography and rechromatography on DEAE-cellulose.

Table 1 also shows that while from 1 mg protein of crude plasma crystalline trypsin releases 0·072 μg of bradykinin, an equivalent amount (mg of protein) of bradykinin-releasing enzyme of the venom released only 0·029 μg. No difference was observed between the fraction precipitated at 0·20 to 0·33 and 0·33 to 0·50 saturation with ammonium sulfate when trypsin was used as bradykinin-releaser, but after chromatography the degree of purification of the substrate was only 43-fold or about one-fourth of that obtained with the enzyme from venom. It should be mentioned that bradykininogen estimations made in the various fractions by the direct method on the ileum gave the same relative activity between trypsin and venom enzyme as that observed by the method of Diniz *et al.*[7]

Fig. 4 shows the assay of bradykininogen performed with the fraction corresponding to the most active peak of the rechromatography. It can be seen that none of the

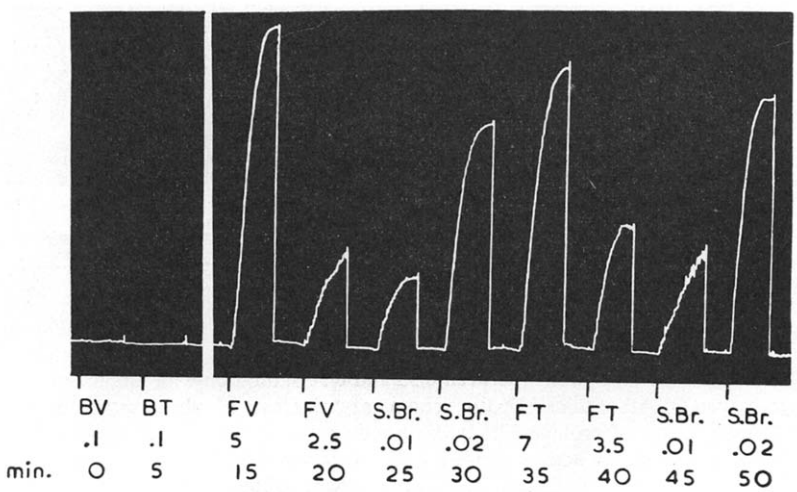

FIG. 4. Bradykininogen content (assayed by the method of Diniz *et al.*[7]) of the most active fraction (F) obtained by re-chromatography on DEAE-cellulose (same conditions as in Fig. 1). Comparison made between single and double doses of synthetic bradykinin (S.Br., in μg) and the following incubated at 37 °C: FV and FT = 14 μg of F (expressed as protein) + 200 μg of venom enzyme or crystalline trypsin, respectively, in a volume of 2·8 ml; BV and BT = 200 μg of venom enzyme or 200 μg of crystalline trypsin in a volume of 2 8 ml. Doses of FV and FT corresponded to micrograms of protein of fraction F; doses of BV and BT, to milliliters of incubate. Each dose remained in contact with the muscle for 90 sec.

enzymes, used in the same proportion as in the incubation mixture, had any action on the ileum. The result of the assay of bradykininogen performed simultaneously in three different horse plasmas with trypsin and bradykinin-releasing enzyme from the venom is shown in Fig. 5.

DISCUSSION

Trypsin or bradykinin-releasing enzyme has been used interchangeably for liberating bradykinin from crude mixtures of plasma proteins.[1] The data presented in this paper,

however, indicate that trypsin and the bradykinin-releasing enzyme from the venom act differently on the same substrate. The following facts were observed: (1) trypsin always released twice as much bradykinin from fresh horse plasma as did the venom enzyme (Fig. 5); (2) in the globulins precipitated with ammonium sulfate a concentration of the venom enzyme substrate was observed in the fraction precipitated between

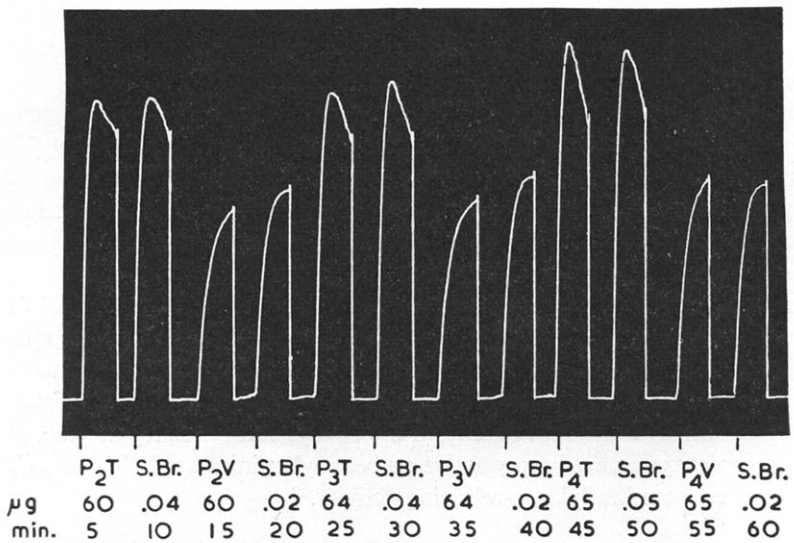

FIG. 5. Bradykininogen content of different horse plasmas (isolated guinea-pig ileum; same conditions as in the previous figures). Incubation at 37 °C of three different horse plasmas (P_2, 1·7; P_3, 1·8; P_4, 1·82 mg of protein) — 200 μg of venom enzyme (V) or 200 μg of crystalline trypsin (T) in a volume of 2·8 ml. Comparison with synthetic bradykinin (S.Br. in μg). Doses of incubates are given in micrograms of plasma protein.

0·33 and 0·40 saturation, while the trypsin substrate was equally spread from 0·20 to 0·50 saturation with ammonium sulfate (Table 1); (3) by chromatography and rechromatography of the globulins precipitated between 0·33 and 0·50 saturation with ammonium sulfate the substrate for the venom enzyme was purified 170-fold in relation to the initial plasma, while the substrate for trypsin was purified only about 40-fold (Table I). Only further work will decide whether the difference of activity observed is owing to the presence of an inhibitor of the bradykinin-releasing enzyme of the venom or to the existence of more than one bradykininogen.

To attain the degree of purification described here for the substrate of the bradykinin-releasing enzyme of the venom, many experiments were performed with other crude substrates as starting material, such as Werle's substrate[3] and untreated fresh plasma. The same technique was used as the one described; i.e. fractional precipitation with ammonium sulfate and chromatography on DEAE-cellulose. In both cases the substrate was eluted with the same buffer concentration as in the chromatography described here. However the degree of purification, observed with the aid of the direct method of assay, was much smaller, since 0·57 mg of protein obtained from Werle's preparation and 0·15 mg of protein from the preparation starting with fresh plasma

were necessary to liberate an amount corresponding to 0·01 μg pf bradykinin, as compared with 0·028 mg of protein of the preparation described here. Horton's substrate[5] is no doubt a better starting material for the purification of bradykininogen. It contains, however, a high bradykinin-releasing activity that must be halted by cold or precipitation with ammonium sulfate as soon as the acidified plasma is neutralized, otherwise the substrate is rapidly consumed. The bradykinin-releasing activity present in Horton's substrate is probably due to plasmin and kallikrein, since both enzymes are stable at pH 2·0[12, 13] and, as shown by Rocha e Silva,[14] the bradykinin-releasing activity of rat's plasma is even enhanced after heating at pH 1·5 to 2·0 for 3–10 min in a boiling-water bath.

The enzyme from the venom of *Bothrops jararaca* was 1·6 times as effective as crystalline trypsin in releasing bradykinin from the purified substrate (Table 1). Since this same enzyme preparation hydrolyzed 6·6 μmoles TAMe/min/mg protein, while trypsin hydrolyzed 82 μmoles TAMe/min/mg protein, it can be concluded that the bradykinin-releasing activity of the venom is independent of its main esterase activity on TAMe. Although the present data do not exclude the possibility that the bradykinin-releasing enzyme from venom has esterase activity, the parallel found by Hamberg and Rocha e Silva[15] between the release of bradykinin and the splitting of BAMe (benzoylarginine methyl ester) by the venom of *Bothrops jararaca* as compared with trypsin, could be owing to the fact that these authors used unfractionated heated venom which contains, apart from the bradykinin-releasing enzyme, *Bothrops* protease A, an enzyme that is very active on benzoylarginine amide[16] and on TAMe[17] while being free from bradykinin-releasing activity.[17]

Acknowledgements—Atropine sulfate was supplied by T. and H. Smith Ltd., London; diphenhydramine hydrochloride by Parke Davis, Detroit; synthetic bradykinin, BRS-640, Sandoz, was obtained through the courtesy of Prof. M. Rocha e Silva, Ribeirão Preto; trypsin, twice-crystallized, salt-free, was supplied by General Biochemicals, Inc., Chagrin Falls, Ohio.

REFERENCES

1. G. P. LEWIS, *Physiol. Rev.* **40**, 647 (1960).
2. M. ROCHA E SILVA, W. T. BERALDO and G. ROSENFELD, *Amer. J. Physiol.* **156**, 261 (1949).
3. E. WERLE, W. GLOTZE and A. KEPPLER, *Biochem. Z.* **289**, 217 (1937).
4. C. G. VAN ARMAN, *Proc. Soc. exp. Biol., N.Y.* **79**, 356 (1952).
5. E. W. HORTON, *J. Physiol., Lond.* **148**, 267 (1959).
6. O. B. HENRIQUES, M. FICHMAN and W. BERALDO, *Nature, Lond.* **187**, 414 (1960).
7. C. R. DINIZ, I. F. CARVALHO, J. RYAN and M. ROCHA E SILVA, *Nature, Lond.* **192**, 1194 (1961).
8. J. H. BURN, D. J. FINNEY and L. G. GOODWIN, *Biological Standardization* p. 210. Oxford Univ. Press, London (1950).
9. E. A. PETERSON and H. A. SOBER, *J. Amer. chem. Soc.* **78**, 751 (1956).
10. H. A. SOBER, J. G. GUTTER, M. M. WYCKOFF and E. A. PETERSON, *J. Amer. chem. Soc.* **78**, 756 (1956).
11. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
12. S. MULLERTZ, *Acta physiol. scand.* **38**, Suppl. 130 (1956).
13. E. WERLE and L. MAIER, *Biochem. Z.* **323**, 279 (1952).
14. M. ROCHA E SILVA, *Polypeptides Which Affect Smooth Muscles and Blood Vessels* p. 210, M. Schachter, Ed. Pergamon Press, London (1960).
15. U. HAMBERG and M. ROCHA E SILVA, *Experientia, Basel* **13**, 489 (1957).
16. O. B. HENRIQUES, A. A. C. LAVRAS, M. FICHMAN, F. R. MANDELBAUM and S. B. HENRIQUES, *Biochem. J.* **58**, 597 (1958).
17. F. R. MANDELBAUM and O. B. HENRIQUES. In press.