ISOLATION AND MOLAR ACTIVITY OF HUMAN BRADYKININ

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Bradykinin released by the action of Bothrops jararaca venom from the bovine plasma was isolated and identified as the nonapeptide H.Arg.Pro.Pro. Gly.Phe.Ser.Pro.Phe.Arg.OH in a previous work (Hamberg, Bumpus and Page 1961). Chemical evidence showed that the molar proportion of amino acids obtained with the natural peptide was identical with that of the nonapeptide. The similar structure was proposed on the basis of the close relationship obtained between the biological activities of the isolated peptide and the synthetic bradykinin (Boissonnas, St. Guttmann and Jaquenoud 1960).

In earlies studies (Hamberg 1959, 1960) the simultaneous activation of plasminogen and release of bradykinin were demonstrated in bovine plasma by using a human urinary activator (Müllertz 1956). The reaction was shown to proceed in parallel with the release of bradykinin obtained in "spontaneously" activated plasma. A comparison between bovine and rat plasma suggested that the spontaneous reaction obtained with acid-heat treated plasma also involved an activation of the fibrinolytic type. These studies have been extended to the human plasma and bradykinin formed in this type of a "spontaneous" reaction after an acid-heat treatment of the plasma substrate has been isolated (Hamberg 1961). Additional data are presented below.

The plasma substrate. The plasma material used in this investigation was comparatively fresh human plasma[x] (ACD + 1/3-1/4 volume EDTA, fresh frozen). The substrate was prepared as described (Hamberg 1959) by an acid-heat treatment to destroy plasmin inhibitors and the bradykinin inactivating enzyme. Plasma diluted with 1/3 - 1/4 volume of saline was brought to pH 1.8-2.0 and heated to 93° by keeping it in a boiling water bath. Dialysis against saline pH 2, 5°, was performed before

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[x] Kindly supplied by the Finnish Red Cross Blood Transfusion Service, Helsinki

using the plasma for incubation. Incubation was performed at 38° after changing the pH of the substrate to 7.6-7.8. Smooth muscle contractile (guinea pig ileum) and hypotensive (cats blood pressure) activity developed slowly and attended a maximum after 3-4 hours of incubation. Corresponding amounts of bradykinin (measured as bovine units) were released per ml of original plasma with fresh (0-21 days) or older (8 month) plasma. Approximately 3 liters of original plasma material was used for obtaining bradykinin released by this "spontaneous" reaction in the substrate. The reaction was stopped by pouring the incubation mixture into 2 volumes of boiling 95 per cent alcohol.

Isolation of released bradykinin. The procedures used for the isolation of bradykinin from total plasma materials have been described before (Hamberg and Deutsch 1958, Hamberg et. al. 1961). The active peptide was eluted from an IRC-50 column (0.8 x 12 cm) in the same pH-range as the bovine bradykinin (pH 6.45-8.00, 75 per cent recovery of activity). The final purification was obtained by paper electrophoresis at pH 8.6, 0.05 M borate, and subsequent paper chromatography with n-butanol, acetic acid and water (4:1:5). For elution of the peptide 5 per cent acetic acid was used The active fraction coincided after electrophoresis and chromatography with the major positive (by Sakaguchi test) peptide fraction. The human bradykinin in a crude form (IRC-50 eluate) moved more rapidly in paper electrophoresis when compared with the synthetic nonapeptide x) and a purified bovine bradykinin. Similar mobility was obtained with the purified human peptide. The Rf values of human and bovine bradykinin coincided; the corresponding active fractions moved ahead of other peptide fractions.

Amino acid analyses. A semi-quantitative evaluation of the amino acid composition was obtained with hydrolyzates (6 N HCl, 16^h, 110^o) of aliquotes from purified peptides fractions in correlation to their respective content of arginine. The purity of fraction obtained by two subsequent purifications with paper chromatography is demonstrated in fig. 1. An amount of peptide corresponding to 7 µg of arginine hydrochloride was hydrolyzed and submitted to two-dimensional paper chromatography in 1) n-butanol, acetic acid and water (4:1:5) and 2) pyridine, acetone and 3 N ammonia (10:6:5). The traces (dotted lines) of other amino acids present except the five contained in bradykinin are (from upper left to low right): leucine or isoleucine, valine, alanine, lysine, glutamic and aspartic acid. This was further

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x) Kindly supplied by Sandoz Inc., Basel, Switzerland.

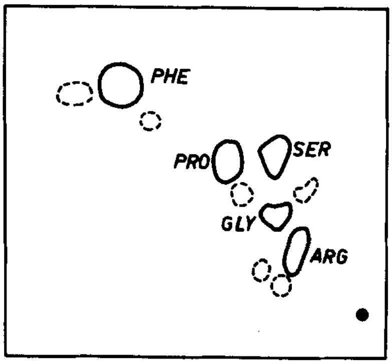

Fig. 1 Diagram showing the amino acids contained in a purified human bradykinin fraction. Two-dimensional paper chromatogram (ascending) developed with ninhydrin.

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sustained by high voltage paper electrophoresis at pH 1.9 with the same amount of amino acids. These contaminating amino acids derived from the adjacent peptide fraction and were not connected with any appreciable amount of activity.

Endgroup analysis. An amount of the peptide fraction corresponding to 14 µg of arginine hydrochloride was reacted with 2:4-dinitrofluorobenzene at pH about 9 ($NaHCO_3$) in approximately 50 per cent alcohol solution for 12 hours. After hydrolysis DNP-arginine was identified by high voltage electrophoresis at pH 1.9 and color reaction from an n-butanol extract (Biserte et al. 1959). The acid ether extract obtained prior to the butanol extraction by shaking the diluted hydrolysate 3 times with ether appeared to contain only dinitrophenol.

Molar distribution of amino acids in the human peptide. An amount of the peptide fraction analyzed in Fig. 1 corresponding to 28 µg of arginine hydrochloride was hydrolyzed and submitted to dinitrophenylation. The DNP-derivatives of the amino acids obtained by chromatography of the ethylacetate extract are shown in Fig. 2. Traces of impurities (dotted lines) appear as di-DNP-lysine, (DNP-methionine ?) and DNP-valine respectively. The control is an aliquote from a DNP-preparation with the synthetic nonapeptide. The amount is chosen so as to correspond to the arginine content of the aliquote used from the human DNP-preparation (about 9 µg arginine hydrochloride). The corresponding spots from the human and control preparations were excised and determined colorimetrically after elution with one per cent sodiumbicarbonate solution. Proline was separated from dinitro-

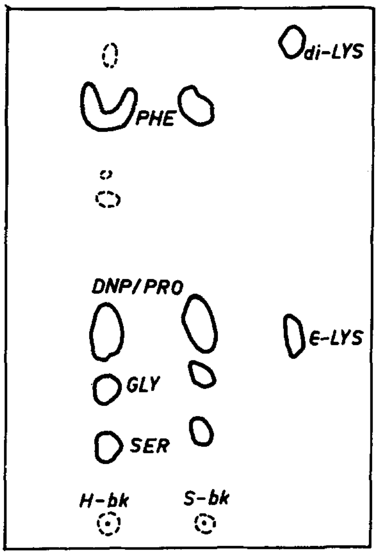

Fig. 2 DNP-derivaties obtained with hydrolyzed human bradykinin (compare fig. 1). Chromatogram 'toluene' from the ethylacetate extract in parallel with a control DNP-preparation of synthetic bradykinin.

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phenol by high voltage paper electrophoresis at pH 3.0. The "DNP + proline' spot was therefore first eluted with methanol for transference to electrophoresis. DNP-arginine was determined separately from the water extract by similar chromatography. Results are presented in Table I.

Table I

Molar distribution of amino acids

	Glycine	Serine	Phenala	Proline	Arginine	n
Synthetic nonapeptide	1.07	1.00	1.87	2.74	2.05	7
Human peptide	1.02	0.85	2.04	3.07	2.03	3

Values are presented per µmole of nonapeptide respectively, as the mean of n chromatographic separations from each DNP-preparation (uncorrected for individual recovery of DNP-amino acids).

Molar activity. The activity of human bradykinin was measured on the isolated quinea pig ileum suspended in a 10 ml bath with oxygenated Tyrode solution at 37-38°. A 3-point assay was applied. A linear dose response was obtained with human bradykinin solutions containing 0, 5, 2, 0 and 4, 0 µg per ml of arginine hydrochloride. The threshold dose in different experiments varied between the range reported for the synthetic nonapeptide (Konzett and Stürmer 1960) corresponding to 1-10 ng/ml of the peptide.

The molar activity was established on the basis of µmoles of arginine content in comparison with the synthetic bradykinin (theoretical amount of arginine). Two solutions containing 4 µg of arginine hydrochloride per ml each (0. 01 µmoles of the pure peptide) were assayed. Parallel and almost identical dose response curves were obtained. The activity ratio U/S (unknown to standard) was 0. 8 as the mean of three dose levels. In a different assay with diluted solutions containing 2 µg arginine hydrochloride per ml the activity ratio was 1, 1. These ratios are in the expected range for this type of assay (Rocha e Silva 1952, Hamberg 1959).

Discussion. These results indicate that the human bradykinin is identical with bovine bradykinin and the synthetic nonapeptide. Although evidence of the amino acid sequence is still missing the similarity is stressed by the molar activity of the peptides as obtained above. This suggests that species differences do not excist between human and bovine bradykinin.

Recent findings that kallidin is a decapeptide form of bradykinin with N-terminal lysine and lower biological activity (Pierce and Webster 1961, Werle et al. 1961, Nicolaides et al. 1961) emphasize the mechanism of release of this type of peptides. The experimental conditions used above differ from those applied by others in that a prolonged heating time (93°) of the acid plasma substrate was chosen. This was done because more bradykinin was obtained at incubation than when the plasma was heated to 56°.

Amino acid analyses of human chromatographic fractions adjacent to bradykinin revealed the presence of lysine which was connected with low activity (about 10 %). A comparison of the distribution of activity obtained in similar paper chromatography by Werle et al. (1961) is of interest. Kallikrein released mainly kallidin which remained in the slower running chromatographic zone adjacent to bradykinin.

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