Methionyl-lysyl-bradykinin, a New Kinin from Ox Blood

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1. The pseudoglobulin fraction from ox serum was maintained at acid pH for several days and was then incubated at pH 7·5 and 37°. At least two polypeptides capable of stimulating smooth muscle were produced and attempts were made to obtain these in the pure state. 2. Only one substance could be purified completely and this was found to be a new plasma kinin, methionyl-lysyl-bradykinin. 3. The biological properties of this new kinin were compared with those of bradykinin and with a sample of synthetic methionyl-lysyl-bradykinin. 4. The possible significance of these results in relation to plasma-kinin formation by kallikrein and other enzymes is discussed.

In a previous paper Elliott, Horton & Lewis (1961) described the isolation of the nonapeptide bradykinin, which was formed by the addition of trypsin to a substrate obtained by ammonium sulphate fractionation of ox serum. It has now been found that this substrate, after being maintained at acid pH under certain conditions, develops the capacity to generate kinin activity on incubation at pH 7·5 without the addition of trypsin. More than one plasma kinin is formed and the present paper describes attempts to purify each of these substances. Only one substance was obtained in the pure state and this was shown to be methionyl-lysyl-bradykinin. The methods of isolation and identification of this peptide are reported, together with a detailed examination of its biological properties. A preliminary account of some of this work has been published (Elliott, Lewis & Smyth, 1963).

EXPERIMENTAL

Materials. The following materials were obtained or prepared for use as described by Elliott *et al.* (1961): $(NH_4)_2SO_4$, ammonium acetate buffers, CM-cellulose, trichloroacetic acid, ether, phenol–water for chromatography, butanol–water for chromatography and glassware was treated with dimethyldichlorosilane. Pyridine–formate buffer, pH 3·25, was prepared as described by Smyth, Stein & Moore (1962). Synthetic bradykinin was generously supplied by Dr E. D. Nicolaides of Parke Davis Laboratories, Ann Arbor, Mich., U.S.A.

Preparation of a mixture of plasma kinins. The crude substrate, prepared from 90 l. of ox blood by $(NH_4)_2SO_4$ precipitation as described by Elliott *et al.* (1961), was dissolved in 10 l. of water, and then 100 ml. of 10 N-HCl was added. The mixture was transferred rapidly to dialysis sacs (Visking) and was dialysed for 3 days against 0·01 N-HCl, which was changed twice daily. Dialysis against distilled water then followed and was continued until SO_4^{2-} was removed. The material remaining in the dialysis sacs, which contained a copious precipitate, was treated with 2 N-NaOH until the pH reached 7·5. The precipitate rapidly dissolved. The clear solution was then incubated at 37° for 6 hr. and at the end of this period it was poured rapidly into 3 vol. of boiling ethanol. Subsequent operations followed the procedure of Elliott *et al.* (1961) and yielded finally a crude mixture of plasma kinins (AP) that corresponded approximately in biological activities to the crude bradykinin described in that paper. The yield from different batches varied between 2 and 7 g.

Trichloroacetic acid treatment of AP. The freeze-dried solid (3 g.) was added in small portions, with stirring of the solution, to aq. 25% (w/v) trichloroacetic acid (20 ml.), which was maintained at 0–5° by external cooling. Gentle stirring was continued until all the solid had dissolved and a heavy brown oil separated from the solution. The oil was separated by centrifugation, then stirred with a second portion of 20 ml. of ice-cold 25% trichloroacetic acid for 30 min. and finally separated by centrifugation. The supernatants were rejected. To the oil were then added 100 ml. of water, 2 ml. of N-HCl and 100 ml. of ether, and the mixture was gently shaken for several minutes. After allowing the layers to settle the ether layer was removed, care being taken not to entrain the heavy oil containing biologically active material. A second portion of ether (100 ml.) was added and the procedure repeated. After two or three such treatments the original three-phase system (heavy oil, ether, water) was converted into a two-phase system of ether and water. A further six extractions with 100 ml. portions of ether were made after the disappearance of the heavy oil to ensure complete removal of the trichloroacetic acid. The aqueous solution was freed of ether by heating it under reduced pressure at 35° for a few minutes, and then freeze-dried to yield the plasma-kinin mixture AP 2 (2 g.).

Chromatography of AP 2 on CM-cellulose. Chromatography of AP 2 (4 g.) was carried out on 32 g. of CM-cellulose with gradient elution exactly as described by Elliott *et al.*

(1961) for crude bradykinin (P2). The three fractions, A, B and C, prepared by combination of the contents of appropriate tubes, were boiled for 2 min., cooled and evaporated to dryness under reduced pressure at approx. 35°. The evaporation was completed in a rotary evaporator and the syrupy residue heated at 50°/0·01 mm. Hg until ammonium acetate had been removed.

Chromatography of peaks B and C on CM-cellulose. This was carried out as described for P5 by Elliott *et al.* (1961). Tubes containing biologically active material were placed in a vacuum desiccator over NaOH and H_2SO_4 until a solid residue remained. They were then placed in a wide glass tube closed at one end and open at the other and fitted with a side arm near the open end. The position of the side arm was above the open ends of the tubes containing the solid. The tube was stoppered, evacuated to approx. 0·01 mm. Hg and immersed as far as the side arm in a bath of water at 50°. Heating under these conditions was continued for 1 hr.

Chromatography of peptides on Dowex 50 (*X* 2). Dowex 50 (X2; 200–400 mesh) was washed successively with N-NaOH, N-HCl and water and was then equilibrated with 0·2 M-pyridine–formate buffer, pH 3·25. The column (15 cm. high × 0·9 cm. diam.) of resin was operated at 50°. Fractions of volume 1 ml. were collected. The gradient was from 0·2 M-pyridine–formate buffer, pH 3·25, to 4 M-pyridine–acetate buffer, pH 5·3, the mixer volume being 150 ml. Peptides were obtained from column fractions by placing the tubes in a vacuum desiccator containing conc. H_2SO_4 and NaOH pellets until all buffer had been removed.

Chromatography of peptides on Sephadex columns. Sephadex G-25 was used in columns (150 cm. × 0·9 cm.) and the eluent was acetic acid–water (1:1, v/v) mixture. Fractions of volume 1 ml. were collected.

Electrophoresis. This was carried out on Whatman no. 1 filter paper that had been washed by descending solvent flow with 2 N-acetic acid for 3 days and then with water for 1 day. The buffer used was 2 N-acetic acid and the voltage gradient was 25 v/cm.

Chromatography. Butanol–acetic acid was prepared by shaking together butan-1-ol, water and acetic acid (63:27:10, by vol.) and keeping for several days before using the upper layer.

Amino acid analysis. Samples were hydrolysed in evacuated tubes in 6 N-HCl according to the method of Crestfield, Moore & Stein (1963), and analyses were kindly carried out for us by Dr S. Jacobs.

Dinitrophenylation of peptides and chromatography of DNP-amino acids. The techniques used were those described by Elliott *et al.* (1961). Water-soluble DNP-amino acids were extracted into butanol after ether extraction of other DNP-amino acids. DNP-arginine and ε-DNP-lysine were distinguished by chromatography in butanol–acetic acid and by application of the Sakaguchi reaction (Jepson & Smith, 1953).

Edman degradation of the peptide from peak C. The technique was essentially that described by Fraenkel-Conrat, Harris & Levy (1955). An autotitrator was used to maintain pH 9 during the reaction of the peptide with phenyl isothiocyanate. Instead of dioxan as described by Fraenkel-Conrat *et al.* (1955) ethanol was used in the reaction mixture. Cyclization of the phenylthiocarbamoyl derivatives was carried out in aqueous 3 N-HCl at room temperature at the first two stages and at 37° at the third stage. The course of the cyclization was studied by scanning the solution at 10 mμ intervals over the range 235–275 mμ until changes in the extinction of the solution, particularly in the region 255–275 mμ, had ceased or were very small. The phenylthiohydantoin derivatives were examined and estimated in the chromatographic systems of Sjöquist (1960). The biological activity of the peptide mixture at each stage of the degradation was compared with that of synthetic bradykinin on guinea-pig ileum or rat uterus.

Digestions with trypsin and chymotrypsin. These were carried out in 0·05 M-ammonium acetate–NH_4HCO_3 buffer, pH 7·5. Incubation temperatures were 37°, and at the end of the digestions a few drops of acetic acid were added to terminate enzymic action. The solutions were evaporated to dryness in a vacuum desiccator over NaOH and H_2SO_4. Substrate:enzyme ratios were approx. 150:1 for trypsin and 50:1 for chymotrypsin. The amount of peptide used was generally 0·25 μmole dissolved in 0·5–1 ml. of buffer solution.

Biological assays. The biological estimation of samples was made by assay on the isolated guinea-pig intestine against a standard sample of P2 (Elliott *et al.* 1961). Results were expressed in terms of the weight of P2 to which the test sample was equivalent in biological activity. The terminal portion of the guinea-pig ileum was suspended in 15 ml. of oxygenated Tyrode solution and maintained at 34°. Contractions of the tissue were recorded by means of a frontal lever writing on a smoked drum of a kymograph.

Actions on isolated smooth-muscle preparations. (*a*) Guinea-pig ileum. A 3–4 cm. segment of terminal ileum from animals weighing 250–400 g. was suspended in a 10 ml. bath containing oxygenated Tyrode solution at 34°. Tests were made every 4 min. and the contact time was 45 sec.

(*b*) Rat uterus. Virgin rats weighing 120–200 g. were injected subcutaneously with stilboestrol (10 μg./100 g.) 16–18 hr. before use. A 2 cm. segment of uterine horn was suspended in a 10 ml. bath containing de Jalon's solution at 30°. Tests were made every 4 min. and the time of contact was 34 sec.

(*c*) Rat duodenum. The proximal 3 cm. of duodenum from rats weighing 120–200 g. was suspended in a 10 ml. bath containing de Jalon's solution at 30°. Tests were made every 3 min. and the contact time was 30 sec.

(*d*) Rat colon. The ascending colon from rats weighing 120–200 g. was suspended in a 10 ml. bath containing de Jalon's solution at 30°. Tests were made every 10 min. and the contact time was 3 min.

Rabbit blood pressure. Rabbits weighing 2–3 kg. were anaesthetized with intravenous urethane (1·25 g./kg.), given as a 25% solution. The trachea was cannulated and the blood pressure recorded from the carotid artery. Intravenous injections were made into the cannula tied into the external jugular vein.

Bronchoconstriction. The method described by Konzett & Rössler (1940) was used in guinea pigs weighing 500–750 g. Injections were made intravenously through a cannula in the superior vena cava.

Vasodilatation. Vasodilatation was examined in the hind limb of cats anaesthetized with chloralose (60 mg./kg.). The preparation was the same as that described by Lewis (1958).

Vascular permeability. This was examined by the method of Miles & Miles (1952). Guinea pigs and rabbits were

injected intravenously with pontamine blue (60mg./kg.). A few minutes after injection of the dye the peptide in 0·1ml. of 0·9% NaCl was injected intradermally into the abdominal skin. After 30min. the area of blueing was measured.

Pain production. Pain production was examined by the method of Armstrong, Dry, Keele & Markham (1953). Blisters were raised on the flexor surface of the forearms of human subjects by the application of cantharidine plasters (2cm.×2cm.). The plasters were applied in the evening before the experiment and allowed to act for 6hr. The area was covered with a sterile dressing and a blister allowed to form during the night. The skin of the blister was cut away and the blister base was washed with a warm Ringer's solution containing (g./l.): NaCl, 9·2; KCl, 0·4; $CaCl_2$, 0·24; $NaHCO_3$, 0·15. Drugs to be tested were dissolved in 0·9% NaCl and kept at 37° during the experiment. The test was carried out as follows.

The solution to be tested was applied to the blister area with a Pasteur pipette until the area was filled. Each dose was allowed to act until the pain reached a plateau or began to subside up to a maximum of 2min. The area was then thoroughly washed with Ringer's solution. Doses were given at intervals of 20min. and before each application the area was washed periodically with Ringer's solution. The subject was not told the nature of the applied solution; he assessed the pain intensity of each solution subjectively, grading it from 0 to +++.

Metabolism. The inactivation of the peptides by plasma kininase was carried out according to the method of Edery & Lewis (1962).

RESULTS

Isolation of methionyl-lysyl-bradykinin

Chromatography of AP2 on CM-cellulose. Bioassays revealed the presence of smooth-muscle-stimulating activity in three separate sections of the chromatogram (Fig. 1), at effluent volumes between 3450 and 3750ml., between 4080 and 4260ml. and again at volumes between 5340 and 5760ml. These were termed peaks A, B and C respectively. Peak A had a total activity equivalent to 1g. of P2, peak B similarly was equivalent to 0·75g. of P2 and peak C was equivalent to 0·75g. of P2. The amount of AP2 added to the column was 3·9g., equivalent in activity to 4·5g. of P2, and the recovery of activity was therefore 55%. The yields of each peak from different batches of AP2 were variable and in some cases one or other was missing altogether.

Chromatography of peaks B and C on CM-cellulose. The results of chromatography of these two substances are given in Fig. 2. (Two separate experiments are incorporated into a single Figure because they were carried out under identical conditions and are therefore comparable on the same base line.) The methionyl-lysyl-bradykinin that formed peak C was obtained in a yield of 6·2mg. from an initial batch of 90l. of ox blood.

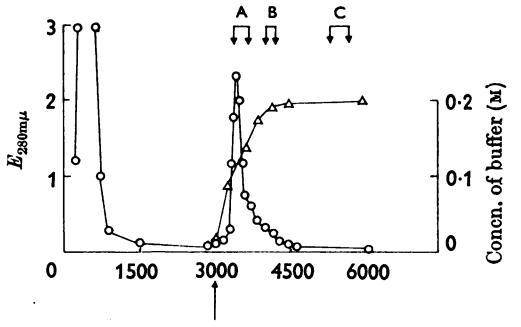

Fig. 1. Chromatogram of 4g. of AP2 on 32g. of CM-cellulose at pH6 in ammonium acetate buffer. The gradient, started at 3000ml. (arrow), was between 0·01M and 0·2M solutions. Biological activity was found at effluent volumes between 3450 and 3750ml. (A), at volumes between 4080 and 4260ml. (B), and again at volumes between 5340 and 5760ml. (C). Fraction A was equivalent in total activity to 1g. of P2, and fractions B and C were each equivalent in total activity to 0·75g. of P2. ○, $E_{280m\mu}$; △, concn. of buffer.

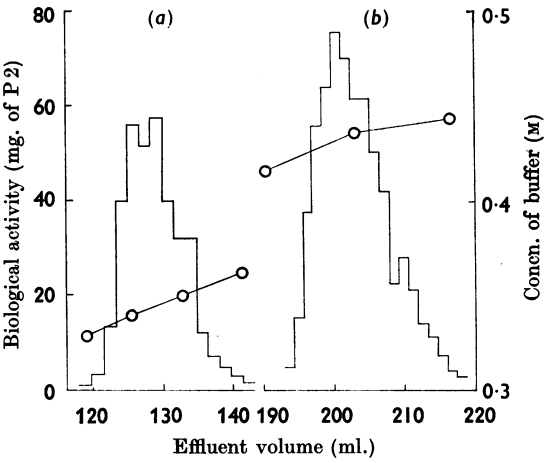

Fig. 2. Chromatograms of peptides from effluents B (*a*) and C (*b*) illustrated in Fig. 1. Individual fraction volumes were 1·6ml. for fraction B and 1·4ml. for fraction C. Biological activity is expressed in terms of the weight of P2 to which the whole fraction would be equivalent in activity. Chromatography was carried out on 4g. of CM-cellulose in ammonium acetate buffer, pH5. The gradient, started at 0ml., was between 0·01M and 0·5M solutions. ○, Concn. of buffer.

The specific biological activity of this peptide was 100 times that of P2.

The position of peak B was close to that for pure bradykinin (Elliott *et al.* 1961), but amino acid

analysis after acid hydrolysis revealed the presence of leucine, isoleucine, tyrosine, valine, alanine, glutamic acid, threonine, aspartic acid and lysine in addition to the amino acids in bradykinin. The weight of peptide obtained from peak B was 9·2mg. and its total biological activity was equivalent to 309mg. of P2, the recovery of biological activity from the column being about 40%. This peptide thus had a low biological activity compared with that of pure bradykinin.

Structural investigations on methionyl-lysyl-bradykinin

The results of amino acid analysis of the pure peptide are given in Table 1 and they show the similarity of the peptide to bradykinin. One residue each of methionine and lysine were present in addition to the total of nine residues in bradykinin. From these values it was calculated that 1 μmole of peptide was present in 1·83mg. of the sample. By means of the 1-fluoro-2,4-dinitrobenzene technique two ether-soluble DNP-amino acids were obtained in low yield and these, when compared with authentic samples, had the same R_F values as DNP-methionine and the corresponding sulphoxide. The water-soluble DNP-amino acid produced was identified as ϵ-DNP-lysine.

A 1·3mg. (0·71 μmole) portion of the peptide was subjected to the Edman degradation. The extinction of the solution of the phenylthiocarbamoyl derivative in 5ml. of 3N-hydrochloric acid at the start of the cyclization was found to be 0·718. The phenylthiohydantoin derivative formed was compared with an authentic sample of methionine phenylthiohydantoin derivative in the solvent system II of Sjöquist (1960) and was found to be identical with it. There were, in addition, two substances of lower R_F in both the authentic sample and the phenylthiohydantoin derivative from the peptide. It was concluded that these were the corresponding sulphoxide and sulphone derivatives because they increased in amount when the samples were exposed to the air. It seemed reasonable to calculate the yield of end group on

Table 1. *Yields of amino acids from a hydrolysate of 0·475mg. of the peptide isolated from peak C (Fig. 2)*

Experimental details are given in the text.

Amino acid	Yield (μmole)
Lysine	0·26
Arginine	0·50
Methionine sulphoxide	0·07
Serine	0·245
Proline	0·78
Glycine	0·27
Methionine	0·13
Phenylalanine	0·48

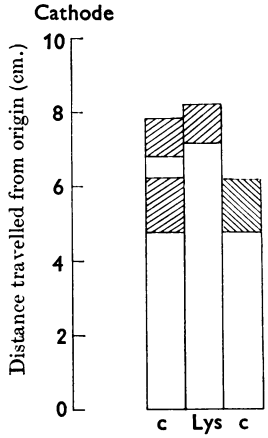

Fig. 3. Result of paper electrophoresis of lysine and of a tryptic digest (c) of methionyl-lysyl-bradykinin at 25v/cm. in 2N-acetic acid. Staining was with ninhydrin (■) and Sakaguchi (▩), reagents.

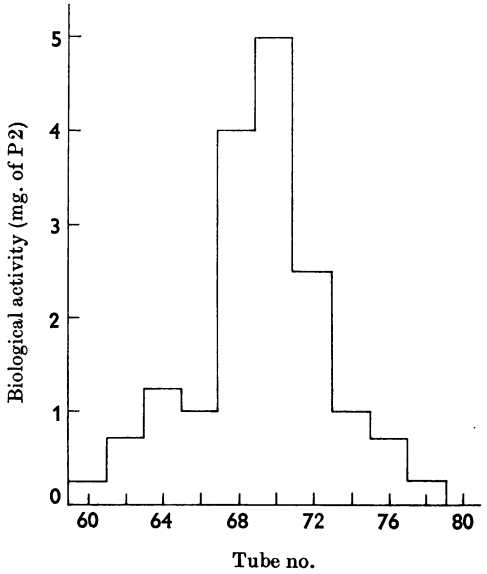

Fig. 4. Chromatogram illustrating the isolation of bradykinin from the tryptic hydrolysate of methionyl-lysyl-bradykinin by using a column (15cm.×0·9cm. diam.) of Dowex 50 (X2). The gradient, commenced at tube 1, was from 0·2M-pyridine–formate buffer, pH3·25, to 0·4M-pyridine–acetate buffer, pH5·3. Bioassays were carried out on alternate tube contents and results are expressed in terms of equivalence to a weight of standard P2. Fractions of volume 1ml. were collected.

the basis of the total amount of phenylthiohydantoin derivative in the three positions on the chromatogram. This was found to be 0·27 μmole, or 38% of the theoretical amount. Two more stages of the degradation were carried out, but at neither stage was it possible to make a definite identification of the phenylthiohydantoin derivatives present. When the peptide was digested with trypsin the biological activity of the solution increased two- to four-fold and was approximately that to be expected if conversion into bradykinin had taken place. Application of the 1-fluoro-2,4-dinitrobenzene technique to the whole digest revealed the presence of an additional end group of arginine in the product, thus indicating the sequence Lys-Arg in the original peptide. Electrophoresis of a tryptic digest, with lysine as a marker substance, gave the results shown in Fig. 3. The faster-moving spot was ninhydrin-positive and Sakaguchi-negative, and yielded lysine, methionine and methionine sulphoxide on hydrolysis. The sequence Met-Lys-Arg had thus been established in the peptide. The slower-moving spot, which was considered to be bradykinin, was positive both to ninhydrin and to Sakaguchi reagents. No attempt was made to isolate the bradykinin from the paper electrophoretogram. In the isolation of bradykinin from the tryptic digest it was found possible to use impure samples of peptide if the isolation was carried out on Dowex 50 (X2). The elution pattern of the biologically active peptide produced is shown in Fig. 4. The amino acid analysis and biological activity of the peptide isolated from the effluent between 60 and 78ml. were identical with those of synthetic bradykinin. Digestion of the pure peptide with chymotrypsin followed by electrophoresis gave the results shown in Fig. 5. Bands 1, 2 and 3 were isolated by the technique of Elliott *et al.* (1961), and after hydrolysis were subjected to paper chromatography. Band 1 consisted of arginine, band 2 contained lysine, arginine, proline, serine or glycine (or both), phenylalanine and methionine, and band 3 contained serine, proline and phenylalanine.

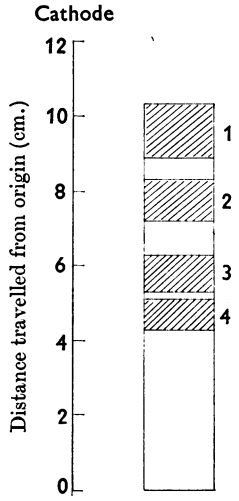

Fig. 5. Results of paper electrophoresis of a chymotryptic digest of methionyl-lysyl-bradykinin at 25v/cm. in 2N-acetic acid. Staining was with ninhydrin.

These results are consistent with the structure:

Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

which is split by trypsin to give methionyl-lysine and bradykinin, and by chymotrypsin to give three products, which are arginine and probably the two peptides Met-Lys-Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe.

The activities, estimated on guinea-pig ileum, of the products were determined at each stage of the Edman degradation. The results (Table 2) show that conversion of the *N*-terminal residue into a phenylthiocarbamoyl derivative brings about an almost complete loss of biological activity in this series of peptides. This is most clearly seen at stages 1 and 2 of the degradation; results at stage 3 were less clear-cut and, as the chemical data also

Table 2. *Biological activity (on guinea-pig ileum) of the crude product at each stage of the Edman degradation of methionyl-lysyl-bradykinin*

(*a*) Activity after formation of the phenylthiocarbamoyl (PTC) derivative. (*b*) Activity after removal of the *N*-terminal residue as a phenylthiohydantoin derivative. Experimental details are given in the text. Results are expressed as percentages of the total activity of the starting material.

Stage of Edman degradation	Biological activity (%)	Structure of peptide
1 (*a*)	1·7	PTC-Met-Lys(ϵ-PTC)-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
(*b*)	78	Lys(ϵ-PTC)-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
2 (*a*)	0	PTC-Lys(ϵ-PTC)-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
(*b*)	19	
3 (*a*)	2·6	
(*b*)	5·2	

demonstrate, this is due to the very poor yields obtained in later stages of the degradation. Quantitative recovery of bradykinin at stage 2(*b*) would have required the biological activity to be about 20 times as great as that found. A phenylthiocarbamoyl residue in the ϵ-position of lysine had very little effect on the biological activity of the decapeptide.

Comparison of the natural methionyl-lysyl-bradykinin with the synthetic peptide

Through the courtesy of Dr E. Schröder, Schering A.-G., Berlin, Germany, we were able to examine the properties of a synthetic preparation of the hendecapeptide (Schröder, 1964). Unfortunately, these comparisons could not be made side by side because the supply of pure natural material had by this time been completely exhausted; it was possible only to compare the synthetic hendecapeptide with synthetic bradykinin as had been done with the natural hendecapeptide. In 2N-acetic acid on washed Whatman no. 1 filter paper the synthetic hendecapeptide migrated at a rate 1·1 times that of synthetic bradykinin. The corresponding ratio for the natural hendecapeptide against synthetic bradykinin was 1·07. Paper-chromatographic comparison was more difficult, owing to the tendency of the peptides to form streaks rather than discrete spots and to the high sensitivity of the R_F values to small changes in the composition of the solvent system. Bradykinin had a higher R_F value than the hendecapeptide in butanol–acetic acid; the ratio of the R_F values of the two synthetic peptides was 1·7, whereas the corresponding ratio for synthetic bradykinin and the natural hendecapeptide was 2·3. During the isolation of the hendecapeptide it was noted that it stained much more strongly than bradykinin with ninhydrin reagent, and this was also observed with the synthetic product.

Biological activity of methionyl-lysyl-bradykinin

In general the biological activities of the pure natural hendecapeptide, as well as the synthetic hendecapeptide, were qualitatively the same as those of bradykinin, although quantitative comparison showed certain differences. Both peptides caused stimulation of isolated smooth-muscle preparations, bronchoconstriction, vasodilatation, increased vascular permeability and pain production. In all preparations where it was possible to study dose–response relationships both peptides gave the same dose–response curve.

Isolated smooth-muscle preparations. Table 3 gives a summary of the results when the natural and synthetic peptides were compared independently with synthetic bradykinin on four isolated smooth-muscle preparations: guinea-pig ileum, rat uterus, rat duodenum and rat colon. On all these isolated preparations, whether the response was contraction or relaxation, bradykinin was 2–5 times as active as either preparation of methionyl-lysyl-bradykinin. The nature of the response of all four tissues was the same to both peptides.

Bronchoconstriction. Both natural and synthetic peptides produced bronchoconstriction when injected intravenously into guinea pigs, just as did bradykinin. As with bradykinin, the guinea pigs showed tachyphylaxis to methionyl-lysyl-bradykinin, desensitization becoming apparent after only one or two injections. Whereas the threshold dose of bradykinin was 0·5–1 μg., with methionyl-lysyl-bradykinin it was 2–4 μg. for the natural and 1·5–3 μg. for the synthetic peptide.

Circulation. The threshold dose of intravenous synthetic methionyl-lysyl-bradykinin required to produce a fall in rabbit blood pressure was 20–50 mμg./kg., i.e. about the same as for bradykinin, although it was found, in agreement with Schröder (1964), that the synthetic hendecapeptide was generally somewhat more active than bradykinin. In spite of the increased relative activity of the hendecapeptide in the rabbit, the hypotensive effect was no more prolonged than that of bradykinin. Fig. 6 illustrates an experiment in which a comparison of the responses at several dose levels shows that synthetic methionyl-lysyl-bradykinin is nearly twice as active as bradykinin. In rats the peptides were found to be equiactive; a depressor response could usually be elicited with 1 μg./kg.

When the natural peptide was compared with bradykinin in the cat it was found that bradykinin was 3–4 times more active in producing vasodilatation in the hind limb. The threshold intra-arterial

Table 3. *Concentration of peptide (mμg./ml.) causing contraction or relaxation of isolated smooth-muscle preparations*

Experimental details are given in the text. C, Contraction; R, relaxation.

	Guinea-pig ileum	Rat uterus	Rat duodenum	Rat colon
Bradykinin	C 0·5–1·0	C 0·1–0·2	R 0·5	R+C 1000
Natural Met-Lys-bradykinin	C 3–4	C 0·3–0·6	R 1·5–2·5	R+C 5000
Synthetic Met-Lys-bradykinin	C 2–4	C 0·2–0·4	R 1–2	R+C 3000

doses were 30–50mμg. for bradykinin and 100–150mμg. for methionyl-lysyl-bradykinin.

Vascular permeability. When injected intradermally into rabbits or guinea pigs that had received pontamine blue intravenously, the hendecapeptide, as well as bradykinin, caused an area of blueing at the site of injection, indicating an increased vascular permeability. The diameters of the blued areas after injections of four different doses of each peptide are given in Table 4. The threshold dose of both natural and synthetic hendecapeptide was the same as that of bradykinin. A response was usually seen after injection of 0·1ml. of a concentration of 0·05μg./ml. in the rabbit and 0·01μg./ml. in the guinea pig. As with the blood-pressure responses in the rabbit, when the dose–response curve was examined the hendecapeptide was somewhat more effective than bradykinin in some experiments.

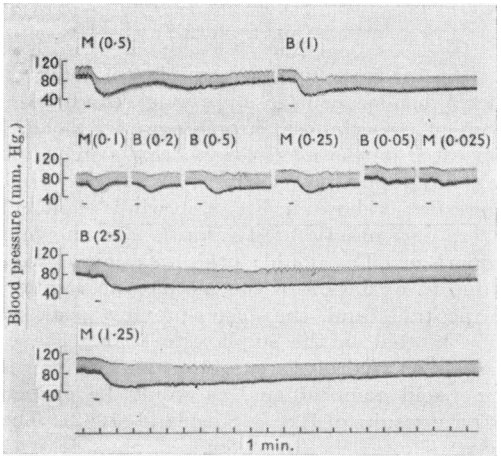

Fig. 6. A rabbit (2kg.) was anaesthetized with urethane (1·25g./kg.) intravenously. The responses of the blood pressure to synthetic bradykinin (B) and synthetic methionyl-lysyl-bradykinin (M) are shown, the doses (in parentheses) being μg./kg.

Pain production in man. Natural methionyl-lysyl-bradykinin, like bradykinin itself, caused pain when applied to an exposed blister base in the human forearm in a concentration of 0·1–1μg./ml. The sensation produced is a burning one like that brought about by application of bradykinin. In addition, after repeated applications of either methionyl-lysyl-bradykinin or bradykinin the blister base rapidly becomes desensitized.

Metabolism. Bradykinin is known to be inactivated when incubated with the globulin fraction of plasma, which contains an enzyme called kininase. Methionyl-lysyl-bradykinin is also destroyed by this enzyme. However, the inactivation is considerably slower with the hendecapeptide. In one experiment, already illustrated in a symposium (Lewis, 1963), a kininase preparation prepared from dog plasma caused little or no inactivation of

Table 4. *Diameter of area of blueing after intradermal injections of peptides into rabbits or guinea pigs that had received pontamine blue intravenously*

Experimental details are given in the text.

Peptide	Animal	Intradermal injection (dose in μg./0·1ml.)	Diam. of area of blueing (mm.) 0·001	0·01	0·1	1·0
Bradykinin	Rabbit		2	6	9	12
			2·5	3·5	8	12·5
			1·5	5	9	11
	Guinea pig		4	4	6	7
			3	7	8	11
			2	3·5	7	7
			3	4	5	7
			1	4	5	7
			2	4	7	9
Natural Met-Lys-bradykinin	Rabbit		1	8	9	11
	Guinea pig		3	6	10·5	13
			2·5	4	8	12
			2	6·5	7	11·5
Synthetic Met-Lys-bradykinin	Rabbit		2	3	5	7
			3	6	8	10
	Guinea pig		3·5	5	6·5	8
			3	5	8	8
			2	4	6	7
			2	4	5	6

natural methionyl-lysyl-bradykinin in 15min., whereas in this time bradykinin was destroyed. The same delayed inactivation has been found with the synthetic hendecapeptide. Fig. 7 illustrates the inactivation of bradykinin and synthetic methionyl-lysyl-bradykinin when incubated with another kininase preparation from dog plasma. A similar relatively slow inactivation of the hendecapeptide occurred when kininase preparations from the plasma of humans and guinea pigs were used.

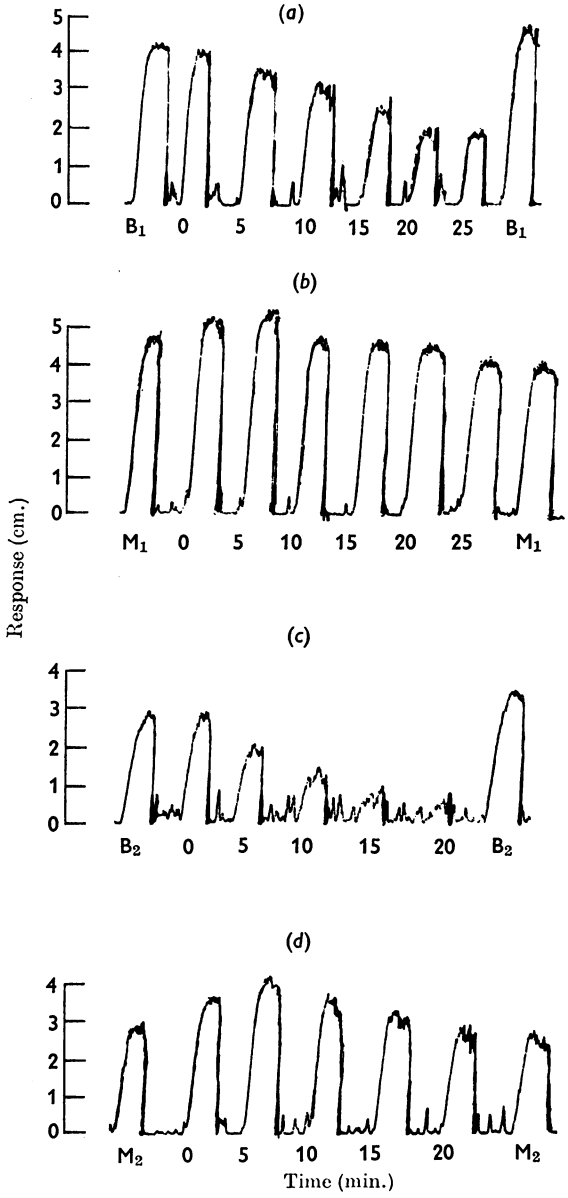

Fig. 7. Responses of guinea-pig ileum to bradykinin (B_1, 5 mμg./ml.; B_2, 2·5 mμg./ml.), to synthetic methionyl-lysyl-bradykinin (M_1, 50 mμg./ml.; M_2, 25 mμg./ml.) and to incubated mixtures of 0·4 μM solutions of bradykinin (in *a* and *c*) and 0·4 μM solutions of methionyl-lysyl-bradykinin (in *b* and *d*) with dog pseudoglobulin (2·5 mg./ml. in *a* and *b*; 5 mg./ml. in *c* and *d*). In each experiment samples were taken and tested at 5 min. intervals from the commencement of the incubation.

Chromatography of methionyl-lysyl-bradykinin on Dowex 50 (X2)

It was frequently not possible to obtain the natural hendecapeptide in a pure condition by means of CM-cellulose chromatography alone. In such cases it was necessary to carry out further chromatography on Dowex 50 (X2). A typical experiment is shown in Fig. 8, in which the elution pattern of synthetic bradykinin is also shown for comparison. The double peak was considered to be due to oxidation of the methionine residue in the peptide, and the faster-moving peak was probably that in the sulphoxide form. The two peptides on hydrolysis exhibited almost identical amino acid compositions, as would be expected from the results of Ray & Koshland (1962). These workers showed that methionine sulphoxide reverts to methionine on acid hydrolysis.

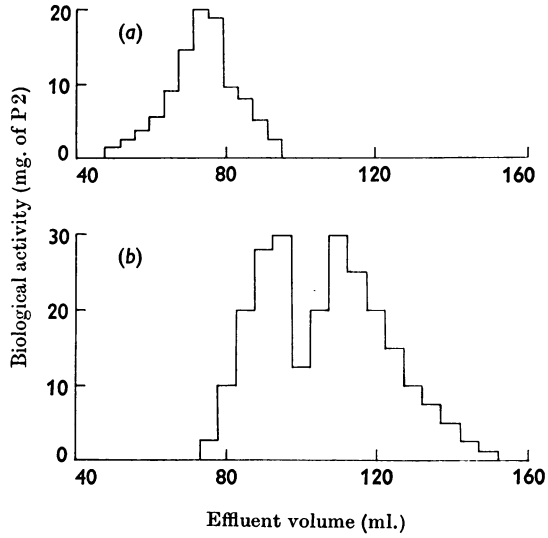

Fig. 8. Chromatograms illustrating the behaviour of synthetic bradykinin (*a*) and the isolation of methionyl-lysyl-bradykinin (*b*) on columns (15 cm. × 0·9 cm. diam.) of Dowex 50 (X2) at 50°. The gradient, commenced at 0 ml., was from 0·2 M-pyridine–formate buffer, pH 3·25, to 4 M-pyridine–acetate buffer, pH 5·3. Biological activity is expressed in terms of equivalence to a weight of standard P2.

Nature of the substances forming peaks A and B on CM-cellulose

When subjected to chromatography on Dowex 50 (X2) both these substances yielded elution patterns that were very similar to that of bradykinin itself. The biological activities of both preparations, as judged by their actions on isolated smooth-muscle preparations, were, however, very low compared with bradykinin, and amino acid analysis revealed the presence of many amino acids in addition to those found in bradykinin. Methionine was absent from the hydrolysates of these peptides, and biological activities and elution patterns from Dowex 50 (X2) of the peptides were unchanged after digestion with trypsin. It was concluded therefore that methionyl-lysyl-bradykinin was absent from these fractions. Peptides isolated from Dowex 50 (X2) after they had been treated with trypsin still contained a large number of amino acids, and their biological potencies had undergone little change. No further attempts were made to purify these substances.

DISCUSSION

This investigation arose during the final stages of the isolation of bradykinin (Elliott *et al.* 1961) when attempts were being made to prepare a more satisfactory substrate for trypsin. It had been found that the yield of bradykinin was sometimes very low and the assumption was made that spontaneous activation of the plasma-kinin-forming system had occurred during the fractionation of ox blood, as indicated by the work of Schachter (1960) and Margolis (1963). The kinins formed would then have been destroyed by kininase or would have passed through the dialysis membrane during the removal of ammonium sulphate from the crude substrate. Acid treatment under various conditions has been widely used as a means of destroying endogenous enzymes before the addition of exogenous plasma-kinin-forming enzymes (e.g. Horton, 1958; Werle, Trautschold & Leysath, 1961; Hamberg, Bumpus & Page, 1961; Pierce & Webster, 1961), and it was considered likely that dialysis of the crude substrate under acid conditions would prevent enzymic activity during this phase of the preparation of bradykinin. This objective was achieved and, further, as incubation experiments at the next stage showed, kininase in the serum was completely destroyed. On the other hand, when the acid-dialysed substrate was neutralized and incubated at pH 7·5 'spontaneous' formation of plasma kinin occurred. It seemed important to attempt the isolation of the pure substance from this endogenous enzymic action, and to compare it with bradykinin. While this investigation was in progress Habermann & Okon (1961) also observed the formation of plasma kinins from an ammonium sulphate fraction of ox serum that had been treated with acid under the conditions of Horton (1958). They reported that the product consisted almost entirely of bradykinin together with a very small amount of the decapeptide, kallidin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), but in more recent experiments (Habermann, 1963) kallidin was obtained in somewhat larger amount. Webster & Pierce (1963) found that incubation of acid-treated human plasma yielded bradykinin and no kallidin.

In view of the fact that chromatographic behaviour is not a satisfactory means of establishing the identity of a polypeptide it was considered essential to isolate all three polypeptides from the incubation mixture and to determine their structures. As shown above, the purification of these peptides proved to be extraordinarily difficult, and for two of them it was abandoned altogether. The hendecapeptide was often contaminated with impurities that were not removed under the most selective conditions that could be devised, but sufficient pure material was obtained for structural analysis. An essential step in the purification process appeared to be the trichloroacetic acid precipitation. It was found that synthetic bradykinin formed an insoluble complex in 25% trichloroacetic acid solution and evidently the hendecapeptide behaved in a similar fashion. The function of this reagent was probably the removal of substances that interfered with subsequent chromatography on CM-cellulose, because no further purification was achieved when this step was eliminated. In the preliminary stages of this investigation the hendecapeptide was confused with kallidin (Elliott, 1963; Lewis, 1963), mainly on account of its chromatographic properties compared with bradykinin and of a coincidence in the results of end-group estimation with 1-fluoro-2,4-dinitrobenzene. Pierce & Webster (1961) reported that no bis-DNP-lysine was obtained from kallidin in spite of its possessing an *N*-terminal lysine residue. The hendecapeptide behaved in a similar way, but this was found to be due to the fact that the end group was a methionine residue and not a 'hindered' lysine residue. The identity of the other peptides formed in these experiments must remain an open question at present. It is possible that they are bradykinin contaminated with persistent impurities, but this could not be established from the present findings. Similarly, the absence of kallidin from these fractions could not be inferred from chromatographic results alone, in view of the possibility that interaction with other peptides could affect the elution pattern. The fact that no change in chromatographic

behaviour was observed when the crude peptides were incubated with trypsin is also not convincing proof of the absence of kallidin, because the *N*-terminal lysine residue of this peptide is removed very slowly by this enzyme (Webster & Pierce, 1963), a finding that was confirmed in these Laboratories with a sample of synthetic kallidin kindly supplied by Dr St. Guttmann of Sandoz laboratories, Basel, Switzerland. It was possible to deduce that a tryptophan residue was absent from the molecule of the hendecapeptide by considering the light-absorption measurements obtained during the Edman degradation. A 0·71 μmole portion of the phenylthiocarbamoyl derivative of the hendecapeptide in 5ml. of 3N-hydrochloric acid had an extinction at 275mμ of 0·72, whereas the contribution from a tryptophan residue alone would have been 0·8 without the contribution from the phenylthiocarbamoyl groups.

A simple modification of the technique of the Edman degradation is worthy of note and particularly concerns the use of an autotitrator to follow the course of reaction of a peptide with phenyl isothiocyanate. Dioxan has been widely used as a solvent in this reaction, but it has been found that it cannot be successfully stored, even over sodium wire, although it may have been obtained absolutely peroxide-free in the first instance. After a short period of storage substances are present (probably peroxides) that bring about a steady production of acidic material, in the presence of phenyl isothiocyanate, at a rate greater than that of the condensation reaction itself. Pure ethanol does not have this property and was satisfactorily used in place of dioxan.

The isolation of the hendecapeptide

Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

raises questions on the specificity of serum kallikrein, which is believed to liberate kallidin from the corresponding substrate. The general consensus of opinion is that serum kallikrein is activated by acid (e.g. Schachter, 1963; Werle, 1963; Webster & Pierce, 1963), and, if both the hendecapeptide and kallidin come from the same substrate, then kallikrein must be capable of splitting the peptide bond between methionine and lysine. In the present experiments no kallidin was found, but Werle *et al.* (1961) isolated kallidin after treatment of a substrate from ox blood with submaxillary kallikrein and did not report the presence of any of the hendecapeptide. Owing to the possibility of species differences in the sequence of the substrate the present experiments cannot be compared with those of Pierce & Webster (1961), who worked with human plasma. It is also possible that more than one substrate for kinin formation is present in plasma (Habermann, Klett & Rosenbusch, 1963; Armstrong & Mills, 1963). As has been discussed before (Elliott, 1963), the fact that acid treatment of plasma leads to activation of endogenous enzyme systems raises serious doubts as to the validity of deductions from experiments in which yet another enzyme is added to the system. The present experiments show that an enzyme with a specificity similar to trypsin would break down the hendecapeptide to bradykinin, and this could be the one and only function of trypsin in the formation of bradykinin itself.

The action of kininase preparations from the pseudoglobulin fractions of various species on the hendecapeptide differed markedly from their action on bradykinin. The hendecapeptide appeared to be destroyed much more slowly than bradykinin, but could have been the resultant of two separate effects: the formation of bradykinin from the hendecapeptide by an enzyme in the globulin with concomitant increase in total activity, and the destruction of bradykinin by the kininase. This possibility has not yet been further investigated. Webster & Pierce (1963) reported that kallidin was rapidly degraded by human plasma to bradykinin by an enzyme that was probably an aminopeptidase.

The fact that the hendecapeptide is formed as the result of activation of endogenous enzyme systems in the body might well mean that the roles ascribed to bradykinin in physiological (Hilton & Lewis, 1957) and pathological (Lewis, 1964) reactions are attributable to methionyl-lysyl-bradykinin.

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