The Solid-phase Synthesis of Some Highly Active Aliphatic Analogues of Bradykinin †

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The synthesis of three analogues of bradykinin is described in which the residues of L-phenylalanine in positions 5 and 8 were separately and together replaced by β-cyclohexyl-L-alanine. The synthesis, on a solid support, utilised the salt-binding principle which led to a significant increase in coupling efficiency in comparison with previous procedures. The analogues formed were of the same order of biological potency as bradykinin itself.

We observed previously[1] that it was possible, by exhaustive hydrogenation, to convert the aromatic rings of the two phenylalanine residues in bradykinin into the reduced form and thereby obtain the [5,8-bis-β-cyclohexyl-L-alanine]-analogue. Further, this analogue appeared to possess smooth muscle-contracting activity to the same degree as the natural local tissue hormone. It was therefore of interest to synthesise this compound in quantity for a more detailed biological investigation, and in addition to prepare, for similar study, the two analogues in which each of the phenylalanine residues is replaced in turn with cyclohexyl-L-alanine. The synthesis of these three compounds by the picolyl ester method is reported in the following paper.[2] While our work was in progress the synthesis of one of these compounds, *viz.* [8-cyclohexylalanine]-bradykinin, was described.[3]

† A preliminary account of this work was read at the 11th European Peptide Symposium, Vienna, May 1971.

[1] D. J. Schafer, G. T. Young, D. F. Elliott, and R. Wade, *J. Chem. Soc.* (*C*), 1971, 46.

It was decided to use the solid-phase technique; in fact bradykinin has frequently been used as a test compound by which the efficacy of variations on the original Merrifield procedure is assessed.[4] Our initial experiments followed the method used by Merrifield for the synthesis of bradykinin,[5] but modifications were introduced in the light of experience obtained. In our hands the esterification of chloromethylated polystyrene (2% divinylbenzene) with t-butoxycarbonylnitro-L-arginine by refluxing in ethanol in the presence of triethylamine gave a low degree of esterification. Resin initially containing 1·26 mg atom of Cl per g was found to contain only 0·09 mmol of nitroarginine per g after 48 h, and this figure was increased only slightly when the reaction time was extended to 65 h.[6] We subsequently

[2] G. A. Fletcher and G. T. Young, following paper.
[3] Ger.P. 1,298,997/1969 (*Chem. Abs.*, 1970, **72**, 44134n).
[4] R. B. Merrifield, *Adv. Enzymol.*, 1969, **32**, 221.
[5] R. B. Merrifield, *J. Amer. Chem. Soc.*, 1964, **86**, 304.
[6] J. M. Stewart and J. D. Young, 'Solid Phase Peptide Synthesis,' Freeman, San Francisco, 1969, p. 32.

used the procedure described by Dorman and Love,[7] which gave loadings sufficiently high for our purpose. It was noted however that of the nitroarginine apparently associated with the resin (0·437 mmol per g), a significant proportion (28%) could be removed by washing with acid. We have assumed that this removable portion was bound ionically to the resin, presumably to unchanged sulphonium groups, and allowance has been made for this in subsequent calculations. This ionic binding phenomenon, though acting as an obstacle at this point, was turned to advantage during later stages of the synthesis.

Initially the procedure described by Merrifield [5] was used to add each amino-acid to nitroarginyl-resin, but it was soon found preferable to replace hydrogen chloride in acetic acid, used for deprotection, by trifluoroacetic acid–dichloromethane (1 : 1),[8] which obviated storage problems and reduced the number of wash sequences in each cycle. Trifluoroacetic acid was removed successfully by washing three times with dichloromethane; use of hydrogen chloride in acetic acid required to be followed by acetic acid, ethanol, and dichloromethane washes to obtain the resin in a form suitable for subsequent treatment with triethylamine to liberate the amino-group of the growing peptide chain.[9]

The most significant modification adopted was at the stage of coupling the protected amino-acid to the growing peptide chain. In the normal procedure a multifold excess of acylamino-acid with an equivalent amount of dicyclohexylcarbodi-imide was shaken with the resin for 3—8 h. The excess of acylamino-acid can be as low as 0·5 equiv.[10] but many workers have used larger amounts. The excess which is not coupled to the growing peptide chain is irrecoverable if, as in this procedure, dicyclohexylcarbodi-imide is used as coupling agent. This loss is a severe disadvantage if the acylating amino-acid is in short supply owing to its being of an unusual nature or radioactive, for example. It was found that the loss of this material could be avoided if use was made of the capacity of the growing peptide with its solid polymeric support to behave as an ion-exchange resin. Basic groups of two kinds occur on the resin: (i) quaternary ammonium or sulphonium groups remaining as a side product of the esterification of the first amino-acid to the resin and (ii) the primary α-amino-groups of the *N*-terminus of the growing peptide chain. It is known that groups of type (i) can bind acylamino-acid.[11] These require to be saturated at each coupling stage; the acylamino-acid bound to them does not take part in any useful reaction and is removed by treatment of the resin with acid at the next deprotection stage. Basic groups of type (ii) might be expected to offer only weak binding sites to carboxylic acids but in practice it seems that binding is sufficiently strong to retain them. The concept of using the growing peptide chain in this way has been mooted previously.[12] No account of its use appeared in the literature until after our work had been completed; Esko and Karlsson [13] then described the effect of salt-binding on yield and racemisation in model systems. These authors confirmed our observations that acylamino-acid binds to the growing peptide chain from dichloromethane solution and only the excess is removed by washing with fresh solvent. During our syntheses the resin was shaken with a three-fold excess of protected amino-acid for 30 min, after which time the resin was filtered off, and the excess of protected amino-acid was recovered from the filtrate. The resin was then shaken with 3 equiv. of dicyclohexylcarbodi-imide for 3 h to effect coupling in the usual way. The overall modified synthetic scheme which was finally adopted is given in Table 1.

TABLE 1

Schedule of operations for one synthetic cycle

Stage [a]	Operation	Reagent added (25 ml)	Shaking time (min)
1	Deprotection	50% TFA [b]–CH_2Cl_2	15
2	Wash (3)	CH_2Cl_2	3
3	Liberation of amino-group	10% NEt_3–$CHCl_2$	15
4	Wash (2)	EtOH	3
5	Wash (3)	CH_2Cl_2	3
6	Amino-acid addition	Boc-AA [c] in CH_2Cl_2	30
7	Coupling	DCCI [d]–CH_2Cl_2	180
8	Wash (3)	CH_2Cl_2	3
9	Wash (3)	EtOH	3
10	Wash (2)	CH_2Cl_2	3

[a] Resin was filtered after each shaking period. [b] Trifluoroacetic acid. [c] t-Butoxycarbonylamino-acid. [d] Dicyclohexylcarbodi-imide.

Hydrogen bromide in trifluoroacetic acid was used to remove peptides from the resin [3] and nitro-protection was removed from arginine by hydrogenation in aqueous 80% acetic acid over palladium–charcoal (5%). By restricting the peptide–catalyst ratio to 3 : 1 w/w or more no reduction of phenylalanine residues to cyclohexylalanine (as judged by subsequent amino-acid analysis) was seen during the time for essentially complete removal of nitro-groups (as judged by electrophoresis). The yield over these two stages was *ca.* 70%. Purification was effected by column chromatography on carboxymethylcellulose in triethylammonium acetate buffer with a pH and molarity gradient.[14] In one case, that of [8-cyclohexylalanine]-bradykinin, a further purification, by counter-current distribution, was necessary in order that the product should give amino-acid analysis results within acceptable limits.

The compounds prepared by the modified coupling procedure required less purification to meet the same

[7] L. C. Dorman and J. Love, *J. Org. Chem.*, 1969, **34**, 158.
[8] S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, 1970, **24**, 1010.
[9] Ref. 6, p. 34.
[10] Ref. 6, p. 24.
[11] H. C. Beyerman, C. A. M. Boers-Boonekamp, W. J. van Zoest, and D. van den Berg, in 'Peptides 1966,' North-Holland, Amsterdam, 1967, p. 117; H. C. Beyerman and R. A. in t'Veld, *Rec. Trav. chim.*, 1969, **88**, 1019.
[12] (*a*) J. Rudinger, personal communication; (*b*) H. Klostermeyer, personal communication; (*c*) W. Lunkenheimer, Doctoral Thesis, Aachen, 1969.
[13] K. Esko and S. Karlsson, *Acta Chem. Scand.*, 1970, **24**, 1415.
[14] J. Porath, *Nature*, 1955, **175**, 478.

criteria of purity than the same compounds prepared by the more usual solid-phase procedure. Chromatography on carboxymethylcellulose sufficed in two cases out of the three examined whereas compounds prepared by the procedure used initially required both column chromatography and counter-current distribution to effect purification. Because of this, yields over this stage were greater when the modified procedure was used, and were *ca.* 70% when column purification alone sufficed. Additional counter-current purification resulted in the mixtures (electrophoresis and t.l.c.), and unequivocal structures have not been allotted to these. Amino-acid analyses were carried out to obtain some clue as to their identity and the interpretation of these was complicated by the repeated occurrence of arginine and proline in the peptide. It was, however, possible to rationalise the data only on the basis of marked foreshortening of peptide chains rather than deletion of residues (with one exception described later). For this reason ion-exchange chromatography was a decisive method of

TABLE 2 †

	Compound	R_F [a] (iv)	(v)	(vi)	R_{Arg} [b] pH 6·1	pH 1·9	$[M]_D$ [c]	Amino-acid ratios [d] Ser	Pro	Gly	Cha	Phe	Arg
(I)	[Cha^{5,8}]-bradykinin	0·33	0·48	0·80	0·61	0·63	−910°	0·86	3·00	0·98	2·02		1·99
(II)	[Cha^{5}]-bradykinin	0·28	0·55	0·77	0·62	0·63	−866	0·84	3·00	0·97	1·00	1·02	1·96
(III)	[Cha^{8}]-bradykinin	0·32	0·42	0·76	0·63	0·64	−899	0·85	3·00	1·00	0·98	0·99	1·95

	Yield [e]	Found (%) C	H	N	Formula [f]	Required (%) C	H	N
(I)	49	53·95	7·55	16·75	$C_{50}H_{85}N_{15}O_{11},3CH_3{\cdot}CO_2H$	53·95	7·35	16·85
(II)	55	53·6	7·9	16·75	$C_{50}H_{79}N_{15}O_{11},3CH_3{\cdot}CO_2H$	53·7	7·8	16·8
(III)	44	48·25	6·05	14·95	$C_{50}H_{79}N_{15}O_{11},3CF_3{\cdot}CO_2H$	48·4	5·95	15·1

[a] Solvent systems are listed in the Experimental section. [b] Electrophoretic mobility. [c] *c* 0·2—0·7. [d] Values relative to Pro = 3·00. [e] Overall based on esterified resin. [f] Compounds were dried to constant weight at 2 mmHg prior to analysis.

† Abbreviations follow those given in (*a*) I.U.P.A.C.—I.U.B. Commission on Biochemical Nomenclature, *Biochemistry*, 1967, **6**, 362; (*b*) I.U.P.A.C. Information Bulletin No. 26, p. 11. Cha = β-cyclohexyl-L-alanine.

TABLE 3

Peptides separated by electrophoresis from chymotryptic digests of bradykinin and its cyclohexylalanine analogues

Fraction * No.	Bradykinin	Amino-acid contents [Cha^{5}]-bradykinin	[Cha^{8}]-bradykinin	[Cha^{5,8}]-bradykinin
1	Ser Pro Phe	Ser Pro Phe	Ser Pro Cha	Ser Pro Cha
2	Pro(2) Gly Phe Arg	Pro(2) Gly Cha Arg	Pro(2) Gly Phe Arg	Pro(2) Gly Cha Arg
3	Arg	Arg	Arg	Arg

* Fractions 1 gave fawn colours with ninhydrin and were Sakaguchi-negative. 2 and 3 gave purple colours with ninhydrin and were Sakaguchi-positive. Fraction 1 R_{Arg} (pH 1·9) 0·49—0·52; 2 R_{Arg} (pH 1·9) 0·67—0·70.

TABLE 4

Amino-acid ratios at various stages in the preparation of [Cha^{8}]-bradykinin by the modified procedure

No.	Compound		Ser	Pro	Gly	NO_2Arg	Phe	Cha	Orn	Arg
1	Peptide attached to resin		0·60	2·95	1·00 *	0·11	0·68	1·18	0·25	1·35
2	After cleavage		0·69	2·71	1·00 *	0·13	0·74	1·14	0·24	1·49
3	After hydrogenation		0·85	2·77	1·00 *		0·82	1·02	0·05	1·77
4	After CMC purification		0·87	2·83	1·00 *		0·78	1·00	0·01	1·98
5	After counter-current		0·85	3·00 *	0·98		0·99	0·98	0·01	1·95
6	By-products separated by column	(I)	0·81	1·25	0·74		0·59	1·00 *		1·06
7	By-products separated by column	(II)	0·68	1·85	0·67		0·51	1·00 *	0·07	1·5
8	By-products separated by counter-current		0·92	2·55	1·05		0·02	1·00 *	0·01	2·1

* Amino-acid taken as reference.

yield over the purification stages being lower, usually 50—55% owing to mechanical losses.

Overall yields, based on amino-acid analysis and calculated on the amino-acid content of esterified resin taken initially, were usually 80—90% after fission from the resin, 70—80% after hydrogenation, and 44—55% after final purification of the free peptide. The products were pure according to electrophoresis and t.l.c. and gave satisfactory results in amino-acid and elemental analyses. The relevant data are given in Table 2.

By-product fractions separated during column and counter-current purification appeared to be complex purification as the enhanced basicity conferred by the second (*N*-terminal) arginine served to move the required product and other peptides containing two arginine residues to a region on the purification gradient well separated from foreshortened peptides containing only the *C*-terminal arginine. In the case where additional counter-current purification was necessary the by-product, separated in this way, gave analytical results close to those required for [desphenylalanine]bradykinin (Table 4, No. 8). The mixtures of foreshortened peptides consisted of chains which had terminated owing to the failure of 4-glycine, 5-phenylalanine, or 6-serine to react.

Our data are consistent with the view that once a chain has failed to react at a given stage it undergoes no further coupling under our conditions.

The behaviour of the cyclohexylalanine analogues towards chymotrypsin digestion was investigated under conditions similar to those described previously for bradykinin itself.[15] The results (Table 3) demonstrated that there was no detectable difference between the analogues and bradykinin in respect of the sites of cleavage by the enzyme. Moreover the conditions used appeared to result in complete cleavage of susceptible bonds in each molecule and no comparison of rates was possible.

[5-, 8-, and 5,8-Bis-cyclohexylalanine]-bradykinins were examined for bradykinin and antibradykinin activity in the following bioassay systems: ileum (guinea pig); uterus, duodenum and blood pressure (rat); hind limb blood flow and nictitating membrane (cat). These experiments showed that all three analogues possessed the same profile of activity as bradykinin and were approximately equipotent with the natural local tissue hormone. It appears therefore, as was indicated previously,[1] that the properties of the phenylalanine side chains associated with their aromaticity can play no significant role in the binding or action of these peptides.

EXPERIMENTAL

Electrophoresis was carried out on Whatman 3MM paper in a Locarte high-voltage apparatus with a voltage gradient of 80 V cm^{-1}. Mobilities are expressed relative to arginine (R_{Arg}). Buffers used were pyridine–glacial acetic acid–water (100 : 10 : 2390 v/v/v) of pH 6·2 and 90% formic acid–glacial acetic acid–water (78 : 148 : 2274 v/v/v) of pH 1·85. Samples for amino-acid analysis were hydrolysed with 6N-hydrochloric acid for 16 h at 115° in an evacuated tube and analysed on a Beckman 120C analyser. When samples contained support resin the hydrolysis medium was 6N-hydrochloric acid–glacial acetic acid (1 : 1). Thin-layer chromatograms were run on Kieselgel G (Merck) or MN 300G cellulose (Macherey, Nagel & Co.). Solvents used for silica gel were (i) $CHCl_3$–MeOH–AcOH (85 : 10 : 5), (ii) $CHCl_3$–AcOH (95 : 5), (iii) Me_2CO–AcOH (98 : 2), (iv) Bu^nOH–pyridine–H_2O (2 : 2 : 1), (v) Pr^iOH–NH_3 aq. (*d* 0·88) (7 : 3); and for cellulose (vi) pyridine–Bu^nOH–H_2O–AcOH (20 : 30 : 24 : 6).

t-Butoxycarbonylamino-acids.—Serine was protected during synthesis by formation of the *O*-benzyl derivative. *O*-Benzyl-DL-serine was acetylated and resolved with α-amylase or acylase I. The L-isomer obtained thereby was converted into the t-butoxycarbonyl derivative by the procedure of Schnabel at pH 9·8.[16] The same procedure, at the appropriate pH, was used for the preparation of t-butoxycarbonyl derivatives of the other amino-acids.

The derivatives of *O*-benzyl-L-serine, cyclohexyl-L-alanine, and phenyl-L-alanine were stored as dicyclohexylammonium salts and generated as required.

t-Butyloxycarbonylcyclohexyl-L-alanine.— Cyclohexyl-L-alanine was acylated according to Schnabel's method at pH 10·2. The reaction was complete after 5 h. The product was isolated as the *dicyclohexylammonium salt*, m.p. 164—166°; analytical sample, m.p. 166—168°, R_F 0·65 (i), 0·48 (ii), and 0·67 (iii) (Found: C, 69·2; H, 10·6; N, 6·45. $C_{26}H_{47}N_2O_4$ requires C, 69·15; H, 10·5; N, 6·2%).

Polymeric Support.—Chloromethylated polystyrene (200 g) (Biobeads SX-2, 200—400 mesh) was fractionated by back-washing with 0·2% BRIJ-35 in 0·25N-sodium chloride. A middle fraction (30—70 μm) was refractionated and gave 75 g of resin (40—60 μm). This was boiled under reflux with 6·7N-hydrochloric acid for 8 h. Chlorine content (microanalysis) was 4·42% indicating 1·26 mg atom Cl per g resin.

Esterification of t-Butoxycarbonyl-N(ω)-nitro-L-arginine to Resin.—(a) *Method of Merrifield.*[6] Esterification was slow and after 48 h at 80° loading of nitroarginine was 90 μmol per g resin (7%).

(b) *Method of Dorman and Love.*[7] Chloromethyl resin (1·26 mmol per g resin) was converted into the dimethyl-(arylmethylene)sulphonium hydrogen carbonate form (450 μequiv. per g) and esterified with t-butoxycarbonylnitro-arginine. After washing with dichloromethane the product contained 437 μmol nitroarginine per g resin. Subsequent washing with N-hydrochloric acid in acetic acid caused this value to drop to 315 μmol per g resin.

Apparatus for Solid-phase Synthesis.—The reaction vessel was of the design described by Merrifield, capacity *ca.* 50 ml. Working loadings were 25 ml liquid and 3 g resin. The vessel was turned through a 90° angle and returned to vertical at a rate of 14 cycles per min (electric motor and gearing).

Reaction Conditions for Solid-phase Synthesis.—(a) *Modified procedure.* Polymeric support (3 g), esterified with t-butoxycarbonylnitro-L-arginine, was introduced into the reaction vessel and taken manually through the schedule of operations given in Table 1 for each amino-acid added. After each shaking period the mixture was filtered before proceeding to the next stage. In stages 6 and 7 the amounts of t-butoxycarbonylamino-acid and of dicyclohexylcarbodi-imide used were 3 equiv. (calculated on the initial resin loading of nitroarginine).

(b) *Merrifield's procedure.* The esterified resin, vessel, and schedule of operations used were essentially as in (a) with the significant difference that stages 6 and 7 were combined. The t-butoxycarbonylamino-acid (3 equiv.) in dichloromethane (25 ml) was added to the resin and, after 10 min shaking, a solution of dicyclohexylcarbodi-imide (3 equiv.) in dichloromethane (5 ml) was added directly without prior filtration of the reaction vessel contents. The reaction was then allowed to proceed.

When required, samples of peptide resin were removed from the reaction vessel after stages 2 and 10 had been completed and subjected to amino-acid analysis.

Cleavage of Peptide from the Solid Support.—Fully protected [Cha[8]]-bradykinin resin (3·6 g; 229 μmol peptide per g resin) was suspended in trifluoroacetic acid (40 ml) at 5° and dry hydrogen bromide was passed through for 30 min. The resin was filtered off and washed thoroughly with trifluoroacetic acid, and the combined filtrate and washings were evaporated to dryness and lyophilised from acetic acid. The *product* (696 μmol) showed one major ninhydrin-positive spot on paper electrophoresis, R_{Arg} (pH 6·1) 0·16; amino-acid ratios are given in Table 4.

[15] D. F. Elliott, G. P. Lewis, and E. W. Horton, *Biochem. Biophys. Res. Comm.*, 1960, **3**, 87.
[16] E. Schnabel, *Annalen*, 1967, **702**, 188.

[8-*Cyclohexyl*-L-*alanine*]-*bradykinin*.—Crude partially protected [Cha^8]-bradykinin (540 mg) was dissolved in 80% acetic acid–water (50 ml), palladium–charcoal (5%; 180 mg) was added, and the mixture was stirred for 30 min. The catalyst was filtered off and washed with 80% acetic acid. A similar weight of fresh catalyst was added to the filtrate, and hydrogenation was conducted at room temperature and pressure until reduction was complete (2—3 days). This was judged by paper electrophoresis at intervals; complete reaction was assumed when a single strong Sakaguchi-positive spot of R_{Arg} (pH 6·1) 0·63 was seen. The catalyst was filtered off and the filtrate evaporated to dryness (310 mg, 381 μmol, 89%). The residue was dissolved in 0·05M-triethylammonium acetate (pH 5·0) and applied to a column (30 × 2 cm) of carboxymethylcellulose (Whatman CM32) which had been equilibrated with the same buffer. The column was developed with a gradient made by running in 0·6M-triethylammonium acetate (pH 7·0) to a mixing flask (250 ml) supplying the column. Fractions (8 ml) were examined by Sakaguchi-positive material and characterised by electrophoresis. Fractions comprising homogeneous products were combined and concentrated. Salt was removed by freeze drying. [8-Cyclohexylalanine]-bradykinin was eluted at a buffer molarity of 0·45. Other compounds eluted at lower molarity [(I) 0·29M and (II) 0·41M] were isolated in the same way. Yields and amino-acid ratios are given in Table 4. The purified [Cha^8]-bradykinin triacetate was homogeneous according to t.l.c. in three systems and to electrophoresis, but the results of amino-acid analysis were outside the limits of experimental error (±3%). The material was dissolved in 10 ml of each phase of the system n-butanol–water–trifluoroacetic acid (50 : 50 : 1), introduced into the counter-current distribution apparatus (Quickfit and Quartz, Steady State model 20) and subjected to 130 upper-phase transfers. Tubes comprising the major peak were combined and lyophilised to give [8-cyclohexylalanine]-bradykinin, homogeneous according to electrphoresis and t.l.c. and giving satisfactory results in amino-acid analysis. Relevant data are given in Table 2.

[5-*Cyclohexyl*-L-*alanine*]- *and* [5,8-*Biscyclohexyl*-L-*alanine*]-*bradykinin*.—These two compounds were prepared in essentially the same way as the [8-cyclohexylalanine]-analogue. Adequate purification was achieved by chromatography on carboxymethylcellulose alone and analytical data for the products, yields, *etc.*, are given in Table 2.

Purification of Peptides prepared by Merrifield's Prodecure.—Following hydrogenation, electrophoresis revealed a more complex mixture of Sakaguchi-positive compounds than was seen when the modified procedure had been used for the synthesis. It was decided to effect a preliminary purification by counter-current distribution by the following method.

Partial Purification of [*Cha*^{5}]-*bradykinin by Counter-current Distribution*.—Crude [Cha^5]-bradykinin (330 mg), after hydrogenation, was dissolved in 10 ml each of the upper and lower phases of the system n-butanol–water–trifluoroacetic acid (50 : 50 : 1) and introduced into the counter-current distribution apparatus. After 200 transfers (upper : lower; 10 : 16) tubes were tested for Sakaguchi-positive material and a wide band was located in tubes 90—120. The contents of these tubes were combined and evaporated to dryness (230 mg, 70%). The peptide was electrophoretically homogeneous [R_{Arg} (pH 6·1) 0·66] but the amino-acid ratios found (see Table 5) were beyond the limits of experimental error for the required [Cha^5]-bradykinin. Further purification was therefore effected by chromatography on carboxymethylcellulose by use of the gradient already described. Recovery over the two purification stages was 43%, and analytical data for the product are given in Table 5.

TABLE 5

Amino-acid ratios at various stages in the preparation of [Cha^5]-bradykinin by the original procedure

Compound	Ser	Pro	Gly *	NO_2Arg	Phe	Cha	Orn	Arg
Peptide-resin	0·60	3·27	1·00	0·16	1·09	1·10	0·25	1·49
After counter-current purification	0·86	2·88	1·00		1·06	1·03	0·02	1·85
After CMC purification	0·86	3·09	1·00		1·04	1·02	0·02	2·02

* Amino-acid taken as reference.

Chymotrypsin Digestion of Cyclohexylalanine Analogues of Bradykinin.—Samples (1 mg) of bradykinin and of each analogue were dissolved in pH 7·5 0·05M-ammonium acetate buffer (4 ml), chymotrypsin (Worthington, three times recrystallised) solution (0·3 ml; 2 mg ml^{-1}) was added, and the mixtures were incubated for 16 h at 37 °C. Glacial acetic acid (0·2 ml) was added and the solutions were concentrated, and applied along a 1 cm length of the start-line marked across a strip (78 × 15 cm) of Whatman 3 MM paper. Arginine was used as a marker and an incubated enzyme blank was applied as a further control. The paper was then subjected to electrophoresis in pH 1·9 formic–acetic acid buffer for 1 h at 80 V cm^{-1}. After drying, the paper was cut into strips and a 2 mm wide portion was removed from each track and used for sample location. Substances were located by spraying with ninhydrin and subsequently with Sakaguchi reagent. After location substances were extracted from the unsprayed paper with hot water then hydrolysed and subjected to amino-acid analysis in the usual way. The mobilities and amino-acid compositions of the various fractions are given in Table 3.

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