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Purification of Bradykinin by Ion-Exchange Chromatography*

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Bradykinin, as prepared by incubation of plasma globulins with the venom of *Bothrops jararaca* (Rocha e Silva, Beraldo & Rosenfeld, 1949), can be assayed upon the guinea-pig ileum, producing a typical contraction of the slow type, not influenced by atropine or anti-histamines. From the crude material, bradykinin is quantitatively extracted into acetic acid, from which it can be precipitated by addition of several volumes of ethyl ether (Prado, Beraldo & Rocha e Silva, 1950). The dry material so obtained is completely soluble in water and can be used as a starting material for further purification. This has been achieved by employing a column of aluminium oxide (Prado *et al.* 1950) and a cellulose (paper pulp) column, according to Helmer (1950), or both (Andrade, Diniz & Rocha e Silva, 1953). We have shown that a combination of the cellulose column and aluminium oxide affords an efficient means of increasing activity of the material, from 4 to 5 'units' per mg. to 1200 'units' per mg.

For reasons given in a previous paper (Andrade, *et al.* 1953), we concluded that bradykinin is a polypeptide; electrophoresis (C. R. Diniz, personal communication) indicated that it has a high isoelectric point (about pH 10) and therefore the application of the carboxylic resin IRC-50 (XE-64) was indicated for further purification, as reported in a preliminary communication (Andrade, 1954).

EXPERIMENTAL

Crude bradykinin preparations. Large batches of ox plasma were precipitated at room temperature with an equal volume of a 50% solution of ammonium sulphate, the precipitate collected in the centrifuge was resuspended in distilled water and dialysed against running tap water for 36–48 hr. The concentrated solution of the globulins so obtained was adjusted to the same volume as that of the original plasma and incubated with venom in 500 ml. lots as described previously (Rocha e Silva *et al.* 1949). The active material was extracted with boiling 70% ethanol and dried. The dried residue was dissolved in a minimum of acetic acid and precipitated with 8 vol. of ethyl ether. The powder was dried over ether and acetone (Prado *et al.* 1950), giving a strongly yellow material, very stable at room temperature. At this stage, about 0·2 mg. of material (equivalent to 1 unit) was obtained from each millilitre of plasma.

Paper-pulp material. About 1·0–1·5 g. of the raw material obtained by precipitation from acetic acid, with an approximate activity of 4–5 'units' per mg., was dissolved in 10 ml. of water added to the top of a column of Whatman no. 1 paper pulp (Helmer, 1950) and 1 ml. of a 80% (w/v) solution of phenol was immediately added with gentle stirring. The details of the procedure for elution and freeze-drying of the active material have been given by Andrade *et al.* (1953). The dry material so obtained has also been used as a standard (PPM-*paper pulp material*) with an activity of 10 'units' per mg. The material so obtained is still yellowish, with a lustrous appearance, and is stored in the ice-box under vacuum in sealed ampoules.

Paper pulp–aluminium oxide material. In a typical experiment, 500 mg. of the dry 'paper pulp material' was dissolved in 5 ml. of 70% (v/v) ethanol and placed in the top of a column (2·5 cm. × 10 cm.) of Brockmann's aluminium oxide (Merck and Co., Rahway, N.J.). About 200 ml. of 70% ethanol was used as the eluting solution. Samples of 1 ml. were collected at about 25 min. intervals with an automatic fraction collector (Technicon, U.S.A.). The samples were analysed for biological activity, for light-absorption at 270 mμ. and for ninhydrin-reacting material, before and after hydrolysis. At this stage the non-hydrolysed material gave consistently negative reactions with ninhydrin.

Amberlite IRC 50 (*XE*-64). Chromatography with this ion-exchange resin was carried out on a 0·9 cm. × 15 cm. column. The preparation of the resin and operation of the column were carried out according to the procedure of Hirs, Moore & Stein (1953). In experiments on rechromatography of the material through the Amberlite column, the eluting solution had a pH higher than that of the column and therefore a gradient of pH was established during elution. This technique has also been used for purifying

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lysozyme (Gonçalves, Molinari & Deutsch, 1956). The rate of flow of solvent through the column was 1·5–2 ml./hr. Sodium phosphate buffer (0·2M) was used as the eluting agent; toluene (1:4000) was added to the buffer as a preservative. Portions of the effluent samples were used for determination of biological activity, for measurement of the absorption at 270 mμ. and for ninhydrin analysis.

Ninhydrin analysis. Estimations of ninhydrin-reacting material were done on 0·2 ml. samples of each fraction, according to the method devised by Troll & Cannan (1953). The samples were hydrolysed in sealed ampoules at 105° for 24 hr. with 6N-HCl. The acid was removed by evaporation *in vacuo* and the residue redissolved by adding 1 ml. of phenol solution, plus 1 ml. of the pyridine solution; the boiling was continued for 3 min. more and the final volume adjusted to 5 ml. with 60% ethanol. Readings were made in a Hilger spectrophotometer at 570 mμ. The results are expressed in millimoles of leucine.

Absorption in ultraviolet light. Fractions from aluminium oxide columns and resin columns showed in the ultraviolet region an absorption spectrum with a maximum at 270 mμ. All readings of the optical densities reported here were made at this wavelength in a Hilger spectrophotometer.

Biological assay. Assays were done on the isolated guinea-pig ileum suspended in Tyrode solution in a 4 ml. bath, as described by Rocha e Silva *et al.* (1949). In such conditions the gut was usually sensitive to 0·2–0·4 biological 'unit'. The indications of activity are given in 'units' of bradykinin, one 'unit' being the activity contained in 1 mg. of 'Pool I', a first homogenized crude preparation (Rocha e Silva *et al.* 1949).

RESULTS

Purification on aluminium oxide. Bradykinin preparations obtained from aluminium oxide columns had a peak of activity corresponding to the maximum of ninhydrin colour after hydrolysis (Andrade *et al.* 1953). By two-dimensional paper chromatography tyrosine was shown to be present among other amino acids. Since large amounts of material had to be chromatographed through the aluminium oxide column, we followed absorption in the ultraviolet region, and found, as shown in Fig. 1, that peaks for biological activity, ninhydrin colour value and absorption at 270 mμ. coincided. About 5000 'units' of bradykinin were passed through a column of aluminium oxide as described, with 70% ethanol as eluent. Small portions of interspaced samples were taken for biological assay and ninhydrin analysis. The yield obtained was about 50% of the original. At this step of purification the material had an average ninhydrin colour value after hydrolysis of $0{\cdot}658 \times 10^{-5}$ m-mole leucine equivalent for one biological 'unit'. One mg. of bradykinin, calculated in leucine equivalents, has 1160 biological 'units'. The active fractions collected from the aluminium oxide column were pooled and the solution was dried in small portions in ampoules and kept under vacuum in the ice-box. The value $0{\cdot}658 \times 10^{-5}$ m-mole leucine equivalent for the material obtained from the aluminium oxide column is about twice the one previously published (Andrade *et al.* 1953). This discrepancy must be due to the standard employed that was found to contain half of the number of 'units' per mg. that were stated in the first publication.

Purification by chromatography on a resin column. A preliminary study was made of the behaviour of a '10 × Pool 1' material, in buffered columns of IRC-50. It was verified that, on elution with sodium phosphate buffer at pH 7·3, the material came out from a 0·9 cm. × 15 cm. column after the passage of about 16 ml. of the effluent solution, and the recovery was about 50% of the original activity. When chromatography was performed with a column buffered at pH 6·58, and eluting solution at pH 7·36, the bradykinin emerged after 100 ml. In

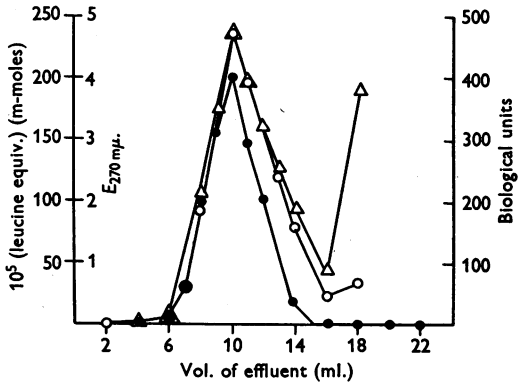

Fig. 1. Chromatography of bradykinin on a 2·5 cm. × 10 cm. column of aluminium oxide. About 500 mg. of 'pulp paper material' was chromatographed. △, Ninhydrin colour value; ○, light-absorption at 270 mμ.; ●, biological activity.

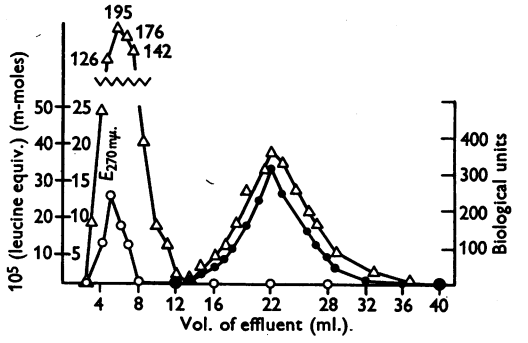

Fig. 2. Chromatography of bradykinin on Amberlite IRC-50. The column was buffered at pH 7·3. The effluent was collected in 0·6 ml. fractions. △, Ninhydrin colour value; ○, light-absorption at 270 mμ.; ●, biological activity.

the latter case the possibility of purification might be higher, but the recovery was lower, about 27% of the original activity. This loss in activity when the effluent volume was increased led us to use the resin column buffered at pH 7·3 and eluent solution at the same pH. About 3200 'units' of bradykinin obtained from the aluminium oxide column (equivalent in colour value to 2·5 mg. of leucine) were dissolved in 1·5 ml. of phosphate buffer at pH 7·3 and applied to the 0·9 cm. × 15 cm. column of IRC-50. It can be seen in Fig. 2 that the biological activity emerges as a peak from 14 to 36 ml., with the maximum at 22 ml. The amount of activity and the ninhydrin colour value are parallel to one another. On the resin column (Fig. 2) it was possible to separate from the active component another ninhydrin-positive material devoid of biological activity. This material collected in the first fractions was found to absorb strongly at 270 mμ. whereas the biologically active fractions did not show any detectable absorption at this wavelength. The passage of another 210 ml. of buffer did not elute any more active material. As in the previous publication (Andrade *et al.* 1953), we have calculated the leucine equivalent per biological 'unit' in each sample of the effluent collected from the column. The values ranged from $0·137$ to $0·158 \times 10^{-5}$ m-mole, with an average of $0·147 \pm 0·002$. The ninhydrin colour value for bradykinin obtained from aluminium oxide column was equivalent to $0·658 \times 10^{-5}$ m-mole leucine per 'unit'; this indicates a fourfold purification of the original material.

Homogeneity of the purest bradykinin preparation. In order to test the homogeneity of the bradykinin preparation, a second resin column was used. The biologically active fractions were pooled, the pH of the solution was brought to 6·8, and the material was fractionated on an IRC-50 column previously buffered at pH 6·8. A solution of phosphate buffer at pH 7·7 was used as eluent. In the experiment of Fig. 3, about 2300 'units' (equivalent to 0·44 mg. of leucine) were employed. The effluent samples collected from the column were submitted to the same routine assay procedures as before. The biological activity came out in a peak at about 66 ml. and the recovery was about 97% of the original.

The symmetrical curves shown in Fig. 3 suggest that the bradykinin is pure, especially since the curve of activity corresponds exactly to the curve of the ninhydrin-reacting material. The high recovery of biological activity observed in this experiment is probably due to the homogeneity of the material employed. Under the same experimental conditions the crude material obtained from an aluminium oxide column would be expected to give a yield of about 35%. For each sample of the effluent collected the leucine equivalent per biological unit was determined. Values ranged from $0·150$ to $0·166 \times 10^{-5}$ m-mole, with an average of $0·155 \pm 0·002$. These values differed very little from those obtained for the material from the first Amberlite column. This agreement might indicate that the value of $0·155 \times 10^{-5}$ m-mole leucine equivalents per biological 'unit' is very near that for pure bradykinin. From these data we can calculate the weight of leucine, in micrograms, equivalent to one biological 'unit' of bradykinin and also the number of biological 'units' contained in 1 mg. of polypeptide calculated as leucine; these figures are included in Table 1.

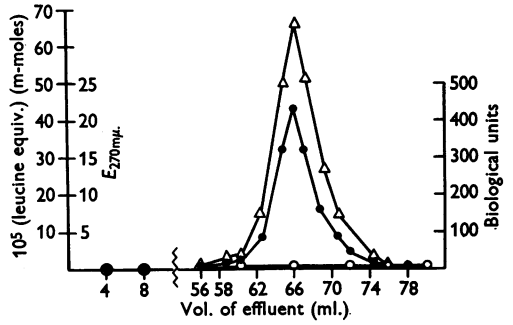

Fig. 3. Chromatography of 2300 'units' of bradykinin on a second column (0·9 cm. × 15 cm.) of Amberlite IRC-50. The effluent was collected in 0·9 ml. fractions. △, Ninhydrin colour value; ○, light-absorption at 270 mμ.; ●, biological activity.

Table 1. *Properties of various samples of bradykinin at different stages of purification*

Material from	10^5 (Leucine equiv./unit) ± S.E. m-moles	Leucine equiv. (μg./'unit')	Biological 'units'/mg. leucine equiv.
(*a*) Paper pulp + aluminium oxide columns	0·658	0·862	1160
(*b*) Resin column, buffered at pH 7·3 and eluent at pH 7·3	0·147 ± 0·002	0·192	5208
(*c*) Resin column, buffered at pH 7·3 and eluent at pH 7·3	0·154 ± 0·01	0·202	4950
(*d*) Resin column, buffered at pH 7·3 and eluent at pH 7·3	0·152 ± 0·006	0·199	5025
(*e*) Resin column, buffered at pH 6·8 and eluent at pH 7·7	0·155 ± 0·002	0·202	4950

The results *a*, *b* and *e* were obtained from experiments reported in Figs. 1–3 respectively. About 1040 biological 'units' were used in Expt. *c* and 2900 'units' in Expt. *d*.

DISCUSSION

The best material obtained in these experiments has an activity of approximately 5025 'units' per mg. of polypeptide (calculated in leucine equivalents). That this ceiling of activity might correspond to pure bradykinin is suggested by the fact that the number of biological 'units' per milligram of polypeptide did not change significantly in both passages through the Amberlite columns.

The present bradykinin obtained from the Amberlite column has a greater activity than histamine, probably much greater if calculated on a molar basis. A small fraction of a biological 'unit' produces a strong contraction of the gut when added to a bath of 4 ml. capacity. About 0·04 μg. of bradykinin produced a reaction of the guinea-pig gut equivalent to that produced by 0·08 μg. of histamine. If it is assumed that the molecular weight is about 5000 (Andrade *et al.* 1953), the potency of bradykinin on a molar basis would be about 80–100 times that of histamine. It has been shown by Gaddum (1955) that the isolated uterus of the rat is more sensitive to bradykinin than is the guinea-pig ileum. In the laboratory of C. A. Keele, at the Middlesex Hospital, in collaboration with Armstrong, one of us (M.R.S.) has shown that the rat uterus is about 25–30 times more sensitive than the ileum. If we assume that the effective concentration of bradykinin to stimulate the guinea-pig ileum is about 0·01 μg./ml., the concentration effective upon the rat's uterus would be about 0·0003 μg./ml., or approximately the same as oxytocin, as it has been found that 500 units of pure oxytocin correspond to 1 mg. (van Dyke, 1955) and that the rat uterus is sensitive to about 0·004 unit/ml. (Holton, 1948).

This preparation was not contaminated with the yellowish material present in the aluminium oxide preparation and the pigment emerged from the Amberlite column in the first effluent fractions. From the results reported in this work we cannot conclude that bradykinin contains no tyrosine since, although there was no measurable absorption at 270 mμ., the amount of pure material used was so small that weak absorption could have been missed. At least we can conclude that the absorption at 270 mμ., shown by the material obtained from the aluminium oxide column, has no relationship to the biological activity, since the absorbing material could be completely separated from the bulk of activity by passage through the Amberlite column. It remains, however, an open question whether bradykinin contains tyrosine in its molecule, and to decide this point much higher concentrations of active material will have to be used. Experiments on two-dimensional paper chromatography with the purified material are in progress in this Laboratory. It has been found that coupling with diazotized sulphanilic acid destroys completely the biological activity (Rocha e Silva, 1951) and this reaction might depend on the presence of either tyrosine or histidine in the molecule. Recently, Vogt (1955) has shown that bradykinin loses its activity after deamination by treatment with nitrous acid. This may indicate the presence of a free α-NH_2 group in the molecule. Also the behaviour of bradykinin in countercurrent procedures indicates, according to Vogt, that a free carboxyl group may be present.

Up to now it has been impossible to obtain a weighed sample of the purest bradykinin for two reasons: any attempt to remove phosphate from the bradykinin with the resins Amberlite IRA-400 and IR-120 failed because bradykinin is also retained by the resins; a few milligrams of this polypeptide corresponds to a large biological unitage, and most of the material obtained from the experiments reported here had to be used for analysis.

SUMMARY

1. Bradykinin can be purified by chromatography on columns of the ion-exchange resin Amberlite IRC-50 (XE-64) buffered with 0·2M solution of sodium phosphate at pH 7·3. When the original material is obtained from a previous passage through an aluminium oxide column, two peaks of ninhydrin-reacting material can be observed in the eluates from the Amberlite column, but only one has biological activity. Absorption at 270 mμ. was absent in the eluates with biological activity.

2. The bradykinin obtained from the Amberlite column may be pure since rechromatography on a new resin column buffered at pH 6·8 with a phosphate solution at pH 7·7 as eluent gave a single ninhydrin-reacting component with about the same biological unitage per milligram (calculated as leucine equivalent) as the original material.

3. The purest bradykinin preparation obtained from these experiments had an activity of 5025 biological units per milligram of polypeptide, calculated as leucine.

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Nitrogen Metabolism in the Sheep

PROTEIN DIGESTION IN THE RUMEN

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The economic importance of ruminants has ensured the continuous investigation of their nutritional requirements, which, it is now clearly recognized, are profoundly influenced by the presence of the rumen. Ingested food is subjected to microbial attack in the rumen of these animals, a process which is essential for the utilization of the roughage which normally forms a substantial part of their diet. Fermentation in the rumen, however, results in the degradation of some of the dietary protein to volatile fatty acids and ammonia, a process which in some circumstances is wasteful when the overall economy of the animal is considered.

Knowledge of protein digestion in the rumen has increased rapidly during the past decade, and an excellent review of the subject has recently appeared (Chalmers & Synge, 1954*a*). The proteolytic activity of rumen contents was recognized by Sym (1938), and Pearson & Smith (1943) obtained evidence for the proteolysis of casein and gelatin incubated with rumen contents. Hoflund, Quin & Clark (1948) found that proteins in fibre form dissolved slowly in the rumen, but the only quantitative measurements of protein breakdown in the sheep rumen are those made by McDonald (1954), who demonstrated that approximately 40% of ingested zein was degraded. Warner (1955) has investigated the proteolytic activity of washed suspensions of rumen micro-organisms, and has studied the degradation of a number of proteins incubated in an 'artificial rumen' similar to that used by Louw, Williams & Maynard (1949). In the presence of toluene, which suppressed the activity of the microbial deaminases, protein was degraded largely to amino acids.

The significance of ammonia as an end product of protein digestion in the sheep rumen was first clearly recognized by McDonald (1948, 1952), who demonstrated enhanced ammonia concentrations in the rumen after the ingestion of certain proteins. Subsequent investigations have confirmed and extended these observations (El-Shazly, 1952*a*; Chalmers, Cuthbertson & Synge, 1954; Annison, Chalmers, Marshall & Synge, 1954). Although proteins are almost certainly converted into free amino acids before degradation to ammonia in the rumen, the concentration of free amino acids is usually low, presumably because of their rapid uptake or degradation (McDonald, 1952). Several workers have investigated the degradation of amino acids by rumen organisms *in vitro*, and to simplify the system to allow the accurate analysis of reaction products the washed-suspension method of Sijpesteijn & Elsden (1952) has usually been employed. Thus El-Shazly (1952*b*), who studied the degradation of casein hydrolysate incubated with washed suspension of rumen organisms, found that ammonia and volatile fatty acids were the principal end products, and the formation of δ-aminovaleric acid from alanine and proline by a Stickland-type reaction was also demonstrated. Sirotnak, Doetsch, Brown & Shaw (1953) and Lewis (1955) have examined the behaviour of individual L-amino acids by the washed-suspension method, but only aspartic and glutamic acids, serine, cystine and arginine were found to be attacked. The dissimilation reactions of aspartic acid incubated with bovine-rumen bacteria were subsequently investigated by Sirotnak, Doetsch, Robinson & Shaw (1954).

In the present work the digestion of protein in the rumen of sheep fed on various diets has been investigated. The rumen ammonia and volatile fatty acid production in sheep fed on similar diets was