'when in addition to phosphoenolpyruvate either alanine, glutamine or aspartic acid was added to the reaction mixture'.

The appearance of $^{14}CO_2$ in certain amino acids during photosynthetic carbon dioxide fixation in a variety of organisms might also be attributed, at least in part, to exchange transamination. Work is at present being undertaken in this laboratory in an attempt to determine the contribution of exchange transamination to the accumulation of label in aspartate by *Chlorella* during photosynthesis and fixation of carbon dioxide in the dark.

SUMMARY

1. In the presence of phosphoenolpyruvate, L-aspartate and an extract of *Kalanchoë crenata*, $^{14}CO_2$ has been introduced into the β-carboxyl group of L-aspartate. The reaction is attributed to an initial carboxylation of phosphoenolpyruvate to yield oxaloacetate followed by an exchange transamination in which aspartate functions as the amino donor.

2. The rate of accumulation of ^{14}C in aspartate has been compared with that of the corresponding reaction, in which the initial carboxylation is followed by a normal transamination in which glutamate serves as the amino donor, and has been shown to be more rapid.

3. The rate of the normal transamination between glutamate and oxaloacetate has been compared with that of the exchange transamination between glutamate and α-oxoglutarate in reactions catalysed by an enzyme from *Ricinus* mitochondria.

4. The results have been discussed in relation to their bearing on $^{14}CO_2$-feeding experiments.

This work was aided by grants from the University of London Central Research Fund and the Department of Scientific and Industrial Research. One of us (R.G.H.) is grateful to the Agricultural Research Council for a studentship. We wish to acknowledge a gift of *Nocardia* enzyme from Dr D. Kerridge, M.R.C. Unit of Microbiology, University of Cambridge. We are grateful to Professor C. P. Whittingham for his advice and criticism.

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The Isolation of Bradykinin, a Plasma Kinin from Ox Blood

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Bradykinin belongs to a group of peptides which stimulate smooth muscle and have been given the generic name of plasma kinin; this name has been adopted by general consent of most workers in the field (see Lewis, 1958). The formation of bradykinin from ox-plasma proteins by the action of trypsin or the venom of the snake, *Bothrops jararaca*, was first observed by Rocha e Silva, Beraldo & Rosenfeld (1949). Blood and many other tissues are known to contain enzymes which can be activated under certain conditions and will then cause liberation of a plasma kinin from the plasma proteins (Frey, Kraut & Werle, 1950). Bradykinin is pharmacologically indistinguishable from other plasma kinins (Lewis, 1958; Gaddum & Horton, 1959; Werle, 1953; Holdstock, Mathias & Schachter, 1957; Mathias & Schachter, 1958; Hilton & Lewis, 1956).

The experiments described in this paper have led to the isolation of bradykinin, which is the first member of the plasma-kinin group to be obtained in the pure state.

EXPERIMENTAL

Materials. $(NH_4)_2SO_4$ was a commercial grade. Ammonium acetate was A.R. grade. Ammonium acetate buffer, pH 5, was prepared from stock solutions of 10M-

ammonium acetate and 10M-acetic acid. The solutions were diluted to the required molarity and then the acetic acid solution was added to the ammonium acetate solution until pH 5 was reached (glass electrode). Approximately 160 ml. of acetic acid soln./500 ml. of ammonium acetate soln. was required.

Carboxymethyl(CM)-cellulose was prepared from Solka Floc cellulose powder by a slight modification of the method of Peterson & Sober (1956). Before its conversion into the carboxymethyl derivative, fine particles were removed from the cellulose by sedimentation. The cellulose powder was stirred with about 6 vol. of water and the mixture was allowed to settle for several hours. Particles which had not settled by this time were rejected by decantation. The process was repeated several times. The sludge was filtered, washed with methanol and dried in air. After the reaction of 60 g. of cellulose with chloroacetic acid and addition of 500 ml. of 10% acetic acid to the reaction mixture, as described by Peterson & Sober (1956), a further addition of acetic acid was made, with stirring, until a pH of 5–6 was obtained. Fine particles were removed by sedimentation as before. It was then transferred to a glass tube 7 cm. × 50 cm., fitted with a filtration plate at the bottom and washed with 10% (v/v) acetic acid until the flame test in the eluate was negative. It was washed with 2 l. of water, transferred to a filter funnel, washed with ethanol and dried in air. The yield was approx. 20 g.

Except in two-dimensional chromatography butan-1-ol was redistilled.

Trichloroacetic acid was A.R. grade, redistilled at 65°/15 mm. Hg.

Ether (*d* 0·720) was allowed to stand for several days over NaOH flakes and redistilled.

Phenol–water for chromatography was prepared by adding 5 vol. of liquid phenol to 2 vol. of water and dissolving in the mixture 0·0003% of ethylenediaminetetra-acetic acid (EDTA).

Butanol–acetic acid for chromatography 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.

Bradykinin at P4 stage and subsequent stages was handled in glassware which had been treated with a solution of dimethyldichlorosilane in carbon tetrachloride and washed with warm water.

Fractionation of ox blood. Fresh ox blood was collected at the slaughterhouse and defibrinated immediately. The red cells were separated by centrifuging in a De Laval Industrial Separator, type 1229G, at room temperature with a gravity disk of 37 mm. internal diam. The rate of feed was 90 l./hr. and the yield of serum was about 44 l. To this amount of serum was added, with mechanical stirring, 22 l. of $(NH_4)_2SO_4$ soln. saturated at room temperature. The ppt. was removed as completely as possible by centrifuging once in a Sharples no. 5 Centrifuge with a 5 mm. diam. jet and the supernatant (60 l.) was treated with 13 l. of saturated $(NH_4)_2SO_4$. Except that a 2 mm. diam. jet was used, the ppt. was collected by centrifuging as before, dissolved in 5 l. of water and dialysed in Visking dialysis tubing against deionized tap water. The latter was prepared with the aid of a Permutit Portable Deminrolit Plant MK4. Dialysis was continued until SO_4^{2-} ion had been removed. The final volume was 9 l.

Preparation of crude bradykinin. The substrate solution, prepared as described above, was treated with 90 ml. of 10N-HCl and then heated at 37° for 30 min. with gentle stirring (cf. Gaddum & Horton, 1959). The pH of the solution was adjusted to 7·5 with 4N-NaOH and incubated for 6 hr. at 37° with crystallized salt-free trypsin (Armour Pharmaceutical Co. Ltd., Eastbourne). The trypsin was added in portions of 75, 37·5 and 37·5 mg. respectively at zero time, 2 hr. and 4 hr. from the commencement of the incubation. The solution was then poured rapidly into 30 l. of boiling ethanol and when the mixture had cooled it was centrifuged and the ppt. was discarded. The supernatant was evaporated under reduced pressure at a temperature not exceeding 40° to a volume of approximately 300 ml. Redistilled butanol was added in small portions to this solution, with gentle shaking, until no more would dissolve. Trichloroacetic acid solution (50%, w/v) was then added to bring the concentration of trichloroacetic acid in the solution to 0·3%. The resulting solution formed the first lower phase of a countercurrent-distribution process carried out in five bottles. The solvent system was prepared by equilibration of equal volumes of butanol and aqueous 1% (w/v) trichloroacetic acid solution. The volumes of upper and lower phases were equal to each other and to the volume of the crude bradykinin soln. forming the lower layer in the first bottle. Emulsions which formed were separated by centrifuging. The process was continued until all bottles were filled with both layers, the upper layer forming the moving phase. The upper phase in the fifth bottle was removed and equilibrations were continued, with addition of a fresh upper phase to the first bottle, until all bottles were again filled. The lower phases in the first two bottles were rejected. The upper phase that had been removed from the fifth bottle was mixed with twice its volume of peroxide-free ether and equal volumes of water and the mixture were equilibrated. The resulting upper layer was rejected and the aqueous phase was combined with the remaining upper and lower phases. The two-phase mixture was evaporated under reduced pressure to a volume of about 200 ml. at a temperature not exceeding 35°. The mixture was fed continuously into a cyclic evaporator, it being so arranged that the upper (butanol) layer formed the major portion of the liquid entering the evaporator until it had all been removed. The aqueous solution was treated with 3N-HCl until pH 1 (glass electrode) was reached and was then extracted nine times with equal volumes of peroxide-free ether, and the ether extracts were rejected. After the fifth extraction the pH of the aqueous layer was readjusted to 1. The aqueous solution was freed of ether by heating it for about 10 min. under atmospheric pressure in a steam bath and then heating it for a few minutes under reduced pressure at about 35°. It was then freeze-dried, yielding a pale-yellow powder. The amount obtained varied considerably, but on average was about 5 g. It was stored in a vacuum desiccator at −2° over NaOH pellets. The crude material (P2) was stable for long periods under these conditions.

Biological assay of bradykinin. The biological activity of samples was estimated by assay on the isolated guinea-pig intestine against a standard sample of P2 which had been set aside at the beginning of this investigation. 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.

Chromatography of bradykinin on carboxymethylcellulose. Columns were run at 20°; they were packed without application of external pressure, but during equilibration the head of liquid used generally caused further settlement. Buffer solutions were saturated with toluene to inhibit growth of micro-organisms. Chromatography of P2 was carried out on 32 g. of CM-cellulose in a column of 3·2cm. diam. through which 0·01 M-ammonium acetate had been passed at approx. 50 ml./hr. until the pH values of the influent and effluent fluids were identical. The pH of the solution was not accurately controlled and varied with different batches of the A.R.-grade ammonium acetate used; it was generally pH 6·4–6·5. A portion (4 g.) of P2 was dissolved in 100 ml. of 0·01 M-ammonium acetate and allowed to run into the column. The column was then washed with 0·01 M-ammonium acetate soln. at the rate of 50 ml./hr. until the extinction of the effluent at 280 mμ was less than 0·02. Gradient elution was then commenced with 0·2 M-ammonium acetate in the reservoir and with a mixing vessel of 250 ml. capacity. In this and other gradient experiments the mixing vessel had only two outlets, one to the column and the other to the reservoir; it had no outlet to the atmosphere. The upper reservoir was open to the air. The effluent was collected in fractions of 30 ml. Fractions containing biological activity were combined, boiled for 2 min., cooled and evaporated to dryness under reduced pressure at a temperature of 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. This product was described as P4.

Chromatography of P4 was carried out on 2 g. of CM-cellulose in a column of 9 mm. diam. which had been equilibrated with 0·01 M-ammonium acetate buffer, pH 5, in the manner already described. A portion (50 mg.) of P4 was dissolved in 0·1 ml. of 0·01 M-ammonium acetate buffer and, after allowing liquid to drain from the top of the column, the solution was added to the column and allowed to soak in. Two successive portions of 0·1 ml. of the buffer were then added in a similar way. The column was then washed with 0·01 M-ammonium acetate buffer, pH 5, which was also 1 mM with respect to EDTA. The hydrostatic head was adjusted to give a flow rate of 5–6 ml./hr and washing was continued until at least 80 ml. of effluent had been collected. The washing fluid was then changed to 0·01 M-ammonium acetate, pH 5; 40 ml. of effluent was collected. Gradient elution was then commenced with a mixing vessel of 100 ml. capacity and 0·5 M-ammonium acetate in the reservoir. Under these conditions the gradient was approximately linear. 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. The combined solid was described as P5.

Chromatography of P5 was carried out on 4 g. of CM-cellulose in a column of 9 mm. diam. It was carried out exactly as for P4 with a mixing vessel of identical capacity.

Electrophoresis of P5. This was carried out on Whatman no. 1 filter paper which had been washed by descending solvent flow with 2 N-acetic acid for 3 days and then with water for 1 day. P5 at a concentration of 10 μg./μl. was applied in the form of a band 2–3 mm. wide and 2 cm. less in length than the total width of the paper. The loading was generally about 100 μg./cm. of paper width; occasionally double this amount was applied with successful results. The buffer used was 2 N-acetic acid and the voltage gradient was 25 v/cm. Electrophoresis was continued for 1 hr. in an apparatus similar to that of Durrum (1950). At the conclusion of the experiment the strip was dried in air at room temperature and a marker strip 0·3 cm. wide was cut lengthwise from the centre of the paper. It was stained with ninhydrin and with Sakaguchi reagent. The section of paper containing the bradykinin was cut out and eluted with water by descending capillary flow, water being allowed to drip from the bottom end of the strip. Elution was generally continued for 16 hr. and the eluate was evaporated to dryness in a vacuum desiccator. The recovery of biological activity was about 70% of that applied to the paper.

Paper chromatography. Two-dimensional chromatograms were carried out with butanol–acetic acid in the first dimension and phenol–water–NH_3 in the second dimension on Whatman no. 1 paper. For one-dimensional chromatography butanol–acetic acid or pyridine–water (80:20, v/v) was used. Chromatograms were dried at 40°. Quantitative amino acid analysis was carried out by the method of Mandelstam & Rogers (1959), a slight modification being made to permit estimation of proline. The chromatogram was developed in butanol–acetic acid on Whatman no. 3 paper, and, after formation of the spots with ninhydrin, all those except the yellow spot of proline were cut out. The remainder of the sheet was moistened by steaming and heated at 110° for 15 min. The yellow spots due to proline became purple and were then cut out. Reproducible results were obtained in the range 0·01–0·1 μM.

Chromatography of dinitrophenyl amino acids (DNP amino acids) was carried out on Whatman no. 1 paper with the *tert.*-pentanol (2-methylbutan-2-ol) system of Blackburn & Lowther (1951) and 1·5 M-phosphate buffer (Levy, 1954), used either singly or for two-dimensional chromatograms. For quantitative work, sections of paper of known area surrounding the spots were cut out, shredded and allowed to stand with 4·5 ml. of 1% $NaHCO_3$ soln. at room temperature for 10 min. Extinction of the extract was measured at 350 mμ, except with DNP-proline, where measurement was made at 370 mμ. Blank estimations were made on pieces of paper cut from the same sheet.

Use of fluoro-2:4-dinitrobenzene for end-group determination and dinitrophenylation of amino acid mixtures. To the peptide or mixture of amino acids (containing between 0·1 and 1 μequiv. of free amino groups), dissolved in a mixture of 1 ml. of 2% (w/v) $NaHCO_3$ and 1 ml. of ethanol, was added 5 μl. of fluoro-2:4-dinitrobenzene. The mixture was shaken by hand for a few minutes, when a homogeneous solution resulted; this was allowed to stand in the dark at room temperature for 3 hr. The solution was then extracted three times with an equal volume of ether. In the determination of end groups the aqueous layer was made

acid (pH 1), evaporated to dryness and hydrolysed with 6N-HCl at 115° for 16 hr. DNP amino acids with the exception of DNP-arginine were extracted from acid solution (pH 1 or less) with ether. If much dinitrophenol was present in the ether extract, this was removed by the vacuum-sublimation technique of Mills (1952). DNP-arginine was extracted with butanol.

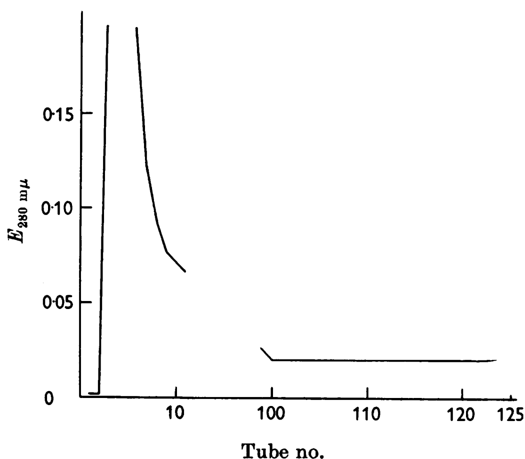


Fig. 1. Results of chromatography of 4 g. of P2 on 32 g. of CM-cellulose at pH 6·4. The fraction volume was 30 ml. The gradient from 0·01 to 0·2M-ammonium acetate was commenced at tube no. 100. Smooth-muscle-stimulating activity was found in tubes nos. 102–125.

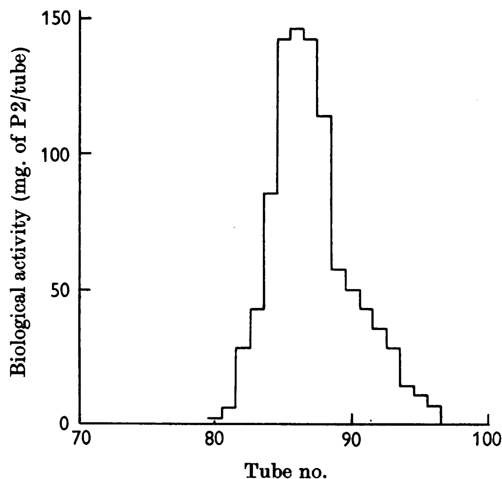


Fig. 2. Results of chromatography of 32 mg. of P4 on 2 g. of CM-cellulose at pH 5. The fraction volume was 1·4 ml. After washing with EDTA (see text) the gradient from 0·01 to 0·5M-ammonium acetate was commenced at tube no. 1. Results are expressed in terms of the wt. of standard P2 to which the tube contents were equivalent in biological activity.

RESULTS

Chromatography of bradykinin on carboxymethylcellulose

Fig. 1 illustrates the results obtained on chromatography of 4 g. of crude bradykinin (P2) on CM-cellulose. It will be seen that a large amount of inactive peptide that absorbs light at 280 mμ emerges in the first 300 ml. of effluent. Further washing with 0·01M-ammonium acetate decreased the extinction of the effluent to about 0·02 at 280 mμ and thereafter it remained almost constant. Tubes nos. 102–125 each contained smooth-muscle-stimulating activity equivalent to more than 5 mg. of P2 and the contents of these were combined. The yield of P4 obtained in this experiment and similar ones was 60–70 mg., which was equivalent in biological activity to 2·4–2·8 g. of P2. The recovery was therefore 60–70%. Inferior results were obtained when chromatography was carried out at 2°.

In Fig. 2 are shown the results of chromatography of P4 on CM-cellulose. The contents of tubes containing biological activity equivalent to more than 10 mg. of P2 were combined. The yield of P5 was 7·8 mg., which was equivalent in biological activity to 925 mg. of P2. An amount of P4 equivalent to 1·4 g. of P2 was used in this experiment and therefore the recovery was 66%.

The results shown in Fig. 3 were obtained on chromatography of 15 mg. of P5 on CM-cellulose. In this experiment there was false indication of incipient separation into two peaks. The contents of tubes nos. 105–110 had the properties of pure bradykinin. The yield was 5·3 mg. It was concluded that the low yield of biological activity in tube 108 was due to a defect in the experimental procedure.

Electrophoresis and paper chromatography of P5

When P5 was submitted to chromatography on Whatman no. 1 paper with butanol–acetic acid as solvent, separation of bradykinin from the inactive peptides was achieved after the chromatogram had been allowed to develop for several days. These results were not sufficiently reproducible to warrant their continued use. At times no separation was achieved and the bradykinin did not leave the origin. It was considered possible that this difficulty was due to contamination with metal ions. Flame-spectrographic analysis (by Johnson, Matthey and Co. Ltd., Wembley, Middx.) revealed that P5 contained 1% by wt. of magnesium. A number of other metallic elements were present, but these were in much smaller quantities than magnesium.

In subsequent experiments paper electrophoresis was used in preference to paper chromatography,

as it was more easily applicable to preparative work. In order to achieve the isolation of pure bradykinin it was necessary to include in the chromatography of P5 a stage of washing with EDTA. Plate 1 shows the results obtained on electrophoresis of P5 prepared with and without the aid of EDTA. It would clearly be impossible to achieve the isolation of pure material from an electrophoresis experiment such as that illustrated in strip no. 1. Strips nos. 2 and 3 show that bradykinin stains weakly with ninhydrin and strongly with the Sakaguchi reagent. By the use of the dipping technique of Jepson & Smith (1953) it was possible to stain the same strip with both ninhydrin and Sakaguchi reagents. The substances stained strongly by ninhydrin on strips nos. 2 and 3 in Plate 1 were biologically inactive.

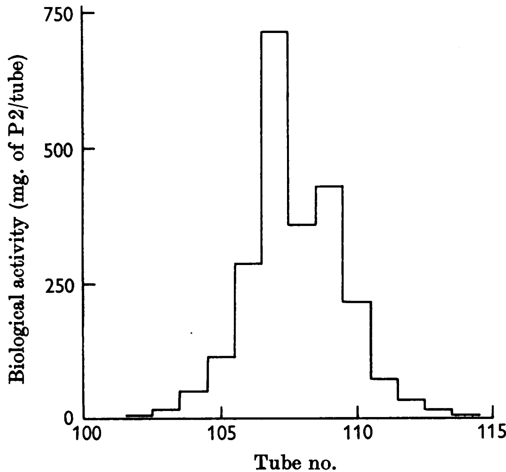


Fig. 3. Results of chromatography of 15 mg. of P5 on 4 g. of CM-cellulose at pH 5. Conditions were identical with those given in Fig. 2.

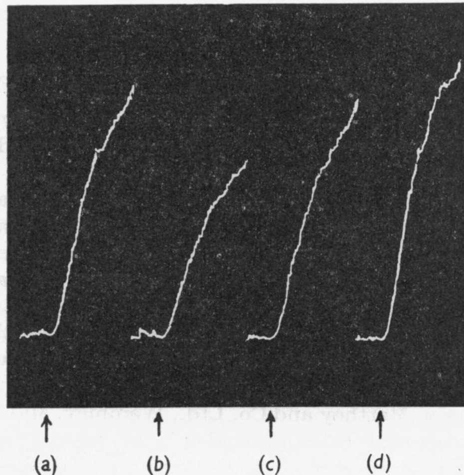


Fig. 4. Responses of the guinea-pig ileum suspended in 15 ml. of Tyrode solution to pure bradykinin (P6), 40 μmg. and 50 μmg. at (*b*) and (*d*) respectively, and to P2, 22·5 μg. and 20 μg. at (*a*) and (*c*) respectively.

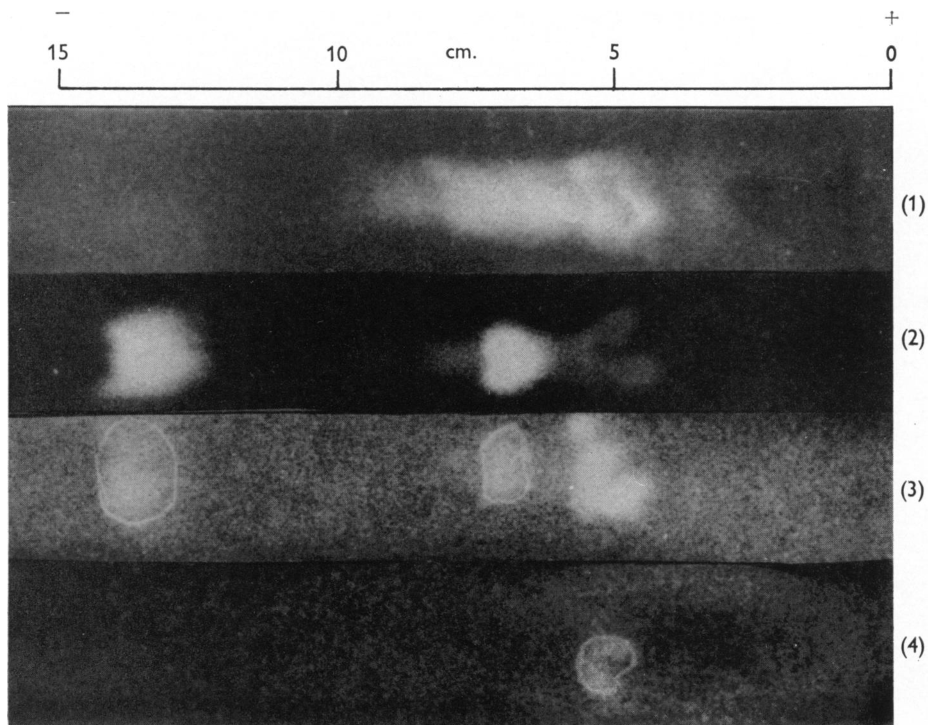
Properties of pure bradykinin

The substance prepared by electrophoresis of P5 as shown in Plate 1 or by further CM-cellulose chromatography of P5 as shown in Fig. 3 was found to contain a single *N*-terminal residue, that of arginine. Hydrolysis followed by two-dimensional paper chromatography revealed the presence of five amino acids, serine, glycine, proline, arginine and phenylalanine. The molar proportions of each amino acid, found by quantitative paper chromatography of the amino acids themselves or of their DNP derivatives, were serine 1, glycine 1, proline 2, arginine 2 and phenylalanine 2. [Degradative work recently carried out by the authors (Elliott, Lewis & Horton, 1960) has revealed that the result obtained for proline is incorrect. The molecule of bradykinin contains three residues of proline.]

Bradykinin was found to possess very high biological potency. For example, the threshold dose for contraction of the isolated guinea-pig ileum was approx. 1 μmg./ml. Fig. 4 shows the results obtained when the biological activity of pure bradykinin (P6) was compared with that of P2. The slow contraction is a characteristic feature of the response of guinea-pig ileum to bradykinin. P6 at a concentration of 3 μmg./ml. produced a marked contraction of the tissue. From these results it was concluded that P6 was 444 times as active as P2. The biological activity of bradykinin was destroyed by chymotrypsin, but not by trypsin. Bradykinin resisted attack by 0·1 N-HCl at 100° for at least 1 hr. and by 0·1 N-NaOH at room temperature for at least 2 hr.

DISCUSSION

The purification of bradykinin on columns of IRC-50 resin was described by Andrade & Rocha e Silva (1956). These authors used phosphate buffers for elution and were unable to remove inorganic ions from the eluates. In the present investigation it was decided that if a volatile buffer could be used this would be much more convenient than a non-volatile buffer. It was decided to explore the potentialities of CM-cellulose in the chromatography of bradykinin. In the course of another investigation, one of us (E.W.H., unpublished work) found that urinary kinin, a peptide closely related to, if not identical with, bradykinin, was irreversibly adsorbed on to IRC-50 in the presence



Results of four separate electrophoresis experiments performed under identical conditions (see text). (1) Electrophoresis of P5 which had been prepared on a CM-cellulose column without EDTA treatment. The strip was stained with ninhydrin. (2) The same technique applied to P5 from a CM-cellulose column with EDTA treatment. (3) Identical with 2, except that the strip was stained with Sakaguchi reagent after ninhydrin. The spots marked by a (pencil) line were positive only to ninhydrin. (4) Electrophoresis of pure bradykinin. The strip was stained with ninhydrin and Sakaguchi reagents.

of ammonium acetate. Losses of activity occurred in all the CM-cellulose columns used and these could have been due to irreversible adsorption.

It was surprising to find that bradykinin contained magnesium after passage twice through CM-cellulose. Accurate data are not available for the magnesium content of pure bradykinin obtained from EDTA-treated columns, owing to the relatively large amount needed for flame-spectrographic analysis. Qualitative results show that it is still present in the purest material, although in decreased amount compared with P 5 prepared without the aid of EDTA.

Recent work has shown (Elliott, Horton & Lewis, 1960) that bradykinin exerts all the actions which have been attributed to one or another of the plasma kinins (smooth-muscle stimulation, vasodilatation, increased capillary permeability, pain production), but the question whether or not the plasma kinins constitute several biologically active peptides or a single peptide (bradykinin) must remain open until other plasma kinins have been isolated and their structures determined.

SUMMARY

1. Bradykinin was formed by trypsin digestion of the fraction of ox-serum proteins which precipitated between 33 and 45 % of saturation with ammonium sulphate.

2. Bradykinin was isolated in the pure state by application of the techniques of countercurrent distribution, chromatography on carboxymethylcellulose and paper electrophoresis.

3. Bradykinin contained the five amino acids glycine, serine, proline, phenylalanine and arginine in the molar proportions 1:1:3:2:2 and one of the arginine residues was *N*-terminal.

The authors are indebted to Mr A. W. Hemmings, who devised techniques for the large-scale fractionation of blood, and to Mr R. Blakemore, Miss M. Kinsella, Mr E. Stevens and Mr I. Croker for valuable experimental assistance.

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Preparation of Purified 3-Hydroxyanthranilic Acid Oxidase from Rat and Ox Liver

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Hepatic 3-hydroxyanthranilic acid oxidase is an enzyme which has been studied hitherto without any previous and proper purification, except the only consistent attempt by Stevens & Henderson (1959), who obtained a fourfold purification from an extract of acetone-dried powder of ox liver.

The present work deals with the conditions for obtaining higher purification of the oxidase. The specific activity we have obtained is 50-fold that obtained by Stevens & Henderson (1959). Some properties of the purified enzyme are also reported.

MATERIALS AND METHODS

Chemicals. 3-Hydroxyanthranilic acid (Hoffman–La-Roche, Switzerland), ethanethiol (Schuchardt, Germany), γ-collidine (Merck, Germany); other products were obtained

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