Br. J. Pharmac. Chemother. (1966), **27**, 256–276.

THE PURIFICATION OF A KININOGEN FROM HUMAN PLASMA

BY

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(*Received January* 10, 1966)

Several groups of workers have described methods for the purification of plasma bradykininogen. Henriques, Picarelli & Ferraz de Oliveira (1962) treated horse plasma with acid, and fractionated by precipitation with ammonium sulphate and chromatography on DEAE-cellulose. The product contained 34% of the bradykininogen present in the acid-treated plasma, and 1 mg of the protein gave the equivalent of 3.14 μg bradykinin with trypsin and 5.0 μg with snake venom.

Habermann & Rosenbusch (1962) purified bovine plasma bradykininogen by Cohn method 6 and chromatography on calcium hydroxylapatite. The product had a specific activity of 10 to 20 μg bradykinin per mg protein.

Greenbaum & Hosoda (1963) purified bovine plasma bradykininogen by ammonium sulphate precipitation, heat coagulation and iso-electric precipitation. The product contained less than 3% of the bradykininogen present in the total plasma globulin and had a specific activity of 0.8 to 1.0 μg bradykinin per mg protein.

Webster & Pierce (1963) purified bradykininogen from stored human plasma by chromatography on DEAE-cellulose and calcium hydroxylapatite. The product contained 15% of the bradykininogen present in stored plasma but further losses occurred during dialysis, filtration and freeze-drying.

The purpose of the present communication is to report a method for the purification of human plasma bradykininogen which has advantages over the earlier methods.

METHODS

The method consists of three main steps.

1. Venous blood was collected and the plasma rapidly separated from the cells. The plasma proteins were equilibrated with phosphate buffer on a column of G-50 Sephadex in preparation for ion exchange chromatography.

2. The part of the eluate which contained the bradykininogen was applied to a column of DEAE-Sephadex. A bradykininogen enriched sample (fraction C) was eluted by stepwise increases in salt concentration. This fraction was suitable for many pharmacological purposes without further treatment.

3. After concentration by freeze drying the bradykininogen of fraction C was purified further by gel filtration on G-200 Sephadex.

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Collection of blood

Blood was withdrawn from the antecubital vein through a wide bore needle and polythene tube into a siliconed, stoppered centrifuge tube graduated at 25 ml. Rapid dispersion of the anticoagulant (500 i.u. heparin B.P. in 0.5 ml. 0.9% NaCl) was ensured by repeated inversion of the tube. After spinning the tube at 2,000 g and 3 to 5° for 15 min. in a refrigerated centrifuge, 10 ml. plasma was withdrawn through a polythene tube into a siliconed syringe lubricated with light paraffin. This was transferred to the G-50 Sephadex column through a fine polythene catheter.

Preparation and use of Sephadex and DEAE-Sephadex columns

1. Coarse G-50 Sephadex (water regain 5.0 g/g dry gel: no. 9860 C) obtained from A.B. Pharmacia (Uppsala), was allowed to swell in buffer for several hr. Fine particles were removed with the supernatant after the bulk of the particles had settled. When this process had been repeated several times the supernatant was clear. The slurry was then exposed to low pressures to remove dissolved air.

The delivery tube of a siliconed glass column was packed with a skein of polythene turnings and an extension tube was fitted to the top. The slurry was poured and the column packed in the way described by Flodin (1962). The upper layer was stirred to ensure a flat surface but no disc was added.

The plasma load was introduced through a 1 mm bore polythene catheter a few mm above the top of the gel. 10 ml. plasma could be layered in this way within 5 to 10 min without disturbance of the gel or mixing with the buffer.

After use the column was dismantled and the process of sedimentation in buffer repeated. The gel was stored in 2 M NaCl at 4°.

2. DEAE-Sephadex, A-50 medium (water regain=5.0 g/g, anion capacity 3.5 ± 0.5 mE/g) was obtained from Pharmacia. Samples from batches 7874 and 1602 were used.

The gel was swollen, freed from fine particles and freed from dissolved air in the same way as the G-50 Sephadex. The slurry was poured and the column packed. Buffer was passed until the *p*H of the eluate was identical with that of the buffer applied.

After use the gel was transferred to a no. 54 Whatman filter in a Büchner funnel. It was stirred with two volumes of 0.5 N NaOH and then washed with distilled water under suction. After stirring with two volumes 0.5 N HCl, the gel was washed with several litres of distilled water followed by buffer. This process produced more fine particles but the subsequent behaviour of the rest of the gel was not altered.

3. G-200 Sephadex (water regain 20.0 g/g dry gel: lot no. 64) was obtained from Pharmacia.

The particles were allowed to swell for at least 72 hr in distilled water containing sodium chloride (about 0.5 M). Particular care was taken during the sedimentation in order to reduce the number of fine particles to a minimum.

The columns were packed on top of a support which consisted of glass wool, 2 mm glass beads and coarse G-50 Sephadex. The top of the column was protected by a filter disc and a 1 cm layer of coarse G-50 Sephadex. These were necessary in order to prevent loads of high specific gravity from displacing loosely packed G-200 Sephadex.

Samples were loaded in the way described for plasma.

Regeneration was carried out by reversing the flow of buffer for several hr after removing the G-50 Sephadex from the top of the column.

Release and estimation of kinin
Materials

(a) Synthetic bradykinin (Bk) Sandoz, BRS 640 Batch no. 0412.

(b) Freeze-dried crystalline trypsin, Tryptar. Armour Pharmaceutical Co Ltd, Eastbourne, England.

(c) Human urinary kallikrein 5.7 Frey units/mg lot V-16-31-bd. (Moriya, Pierce & Webster, 1963).

(d) Hog pancreatic kallikrein, 120 Frey units/mg lot 21 Z-4 (Moriya, Pierce & Webster, 1963).

(e) Uncontaminated human parotid saliva was collected through a fine polythene catheter which drained a Perspex cup placed over the ampulla of the parotid duct. The cup was held securely against the buccal mucosa by applying suction to an annular trough cut around the cup in the Perspex block (Curby, 1953). Salivation was stimulated by applying citric acid and sucrose to the back of the tongue.

(f) Ethanol distilled in glass over calcium oxide.

Estimation of kininogen in whole plasma

0.2 ml. aliquots of plasma were ejected rapidly from a blow-out pipette into 5 ml. 80% ethanol at 0°. The suspended precipitate was heated rapidly to 80 to 90° and held at that temperature for 20 min. The suspension was centrifuged and the ethanol removed by decantation. After washing in distilled water the precipitate was suspended in 0.02 M sodium phosphate buffer, *p*H 7.35, 0.10 M NaCl. Trypsin was added to give a final concentration of 200 μg/ml. and the agitated suspension was incubated at 37° for 20 min. The enzyme was then inactivated by heating at 100° for 10 min. The samples were frozen or assayed immediately. The kininogen content was expressed in units. One unit was that amount which yielded kinin equivalent to 1 μg of synthetic bradykinin, when assayed on the isolated guinea-pig ileum or rat uterus.

Estimation of kininogen after gel filtration

The 50 ml. eluate from the G-50 Sephadex column was allowed to run directly into 200 ml. absolute ethanol. The protein suspension was mixed vigorously and 5 ml. aliquots were withdrawn rapidly. Each was treated exactly as described above.

Estimation of kininogen in purified samples

Protein fractions eluted from the DEAE and G-200 Sephadex colums were incubated with trypsin (60 or 10 μg/ml.) for 5 min unless otherwise stated. No denaturation in ethanol was carried out. The presence of 0.5 M NaCl in some of the gel-filtration experiments did not prevent the release of kinin by trypsin.

The estimation of free kinin

Aliquots were heated to 100° for 10 min immediately after collection in order to denature enzymes which might have been able to release or inactivate kinin. In a few experiments kinin was extracted in hot ethanol. The ethanol was removed at 40° using a vacuum pump and capillary leak.

Biological assay

The kinin released from whole plasma was assayed on the guinea-pig ileum. The greater sensitivity of the rat uterus was essential for the examination of fractions from the chromatographic experiments. The rat duodenum was used to confirm that the kinin released from the purified kininogen was bradykinin-like. It was not used for routine assay.

(a) Guinea-pig ileum

A piece of guinea-pig terminal ileum about 2 cm long was suspended in a 2 ml. bath at 34° to 36°. The tissue was bathed in oxygenated Tyrode solution. A light isotonic frontal writing lever with a six-fold magnification exerted a tension of 0.5 g on the tissue. 45 sec contact between drug and tissue, every four min was usually satisfactory. Mepyramine maleate and atropine sulphate (1 μg/ml.) were used to increase specificity.

(b) Rat uterus

Virgin, albino rats (100 to 150 g) were given a subcutaneous injection of 10 to 15 μg stilboestrol in arachis oil. 18 to 20 hr later they were killed and the uterine horns excised. A length of 1 to 2

cm was suspended in a 1.5 ml. bath containing oxygenated de Jalon solution with atropine sulphate (1 μg/ml.) at 29 to 31°. The recording lever described above was used.

A sample was allowed to remain in contact with the tissue for up to two min. The cycle was repeated every six min. The threshold concentration of bradykinin was commonly about 0.25 ng/ml. The dose-response curve was steep; a concentration of 1.0 to 1.5 ng/ml. often elicited a maximal response.

In the majority of experiments where large numbers of fractions were being screened, the assay was of a simple bracket type. The calculations of the specific activities of the most highly purified preparations of kininogen were however based on 2+1 dose assays and the calculation of fiducial limits.

(c) Rat duodenum

The first 2 to 3 cm of the duodenum was suspended in oxygenated de Jalon solution at 29 to 31°. The fiuid in the 2 ml. bath was changed by overflow but the flow was stopped for up to one min after the addition of a sample. The frontal writing system exerted a tension of 0.5 g and magnified about 10-fold.

Determination of the concentration and physical properties of protein
Measurement of protein concentration

The extinction at a wavelength of 275 mμ was determined using a Unicam SP 500 Spectrophotometer. This was used as an approximate measure of protein concentration.

More accurate determinations were made by the method of Lowry, Rosenbrough, Farr & Randall (1951) which uses the Folin, Ciocalteau reagent. The reagent and sample volumes were doubled to allow the use of cuvettes with a 1 cm light path. No other modifications were made.

Standard calibration curves were prepared with diluted normal human serum. This standard was used for the measurement of protein concentrations in the fractions from the DEAE-Sephadex columns (Table 3).

In later experiments with the purified kininogen eluted from the G-200 Sephadex columns, bovine serum albumin was used as the standard. A standard calibration curve was determined with each new group of measurements.

Estimation of molecular weight

The following proteins were compared with human kininogen on G-200 Sephadex.

(i) Twice crystallized ovalbumin (salt-free) L. Light and Co, Colnbrook, England.

(ii) Bovine serum albumin (fraction V) batch CH 3170, Armour Pharmaceutical Co Ltd, Eastbourne, England.

(iii) Rabbit gamma globulin (fraction II) lot 29, Pentex Incorporated, Kankakee, Illinois, U.S.A.

Electrophoresis

(a) Starch gel

The method (Cruft & Leaver, 1961) was a small scale modification of the original (Smithies, 1955). Starch gel was prepared as described by Smithies from partially hydrolysed starch (British Drug Houses Ltd, Poole, England), and poured on to glass plates. The gel was covered with a polythene sheet and rolled flat.

Samples of Fraction C (see Tables 1 and 2) were concentrated 20-fold by pressure dialysis against 0.03 M borate buffer (*p*H 8.85), mixed into a paste with starch grains and placed in troughs (0.1 × 1.0 cm) cut in the gel. Evaporation was prevented by a coat of low melting point paraffin. After passing a current of 2.0 mA for 10 hr the gel was washed with petroleum ether and divided by a vertical, longitudinal cut. Half was stained with amido black (saturated solution in water-methanol-acetic acid, 1:1:1, v/v/v) and the other half was divided transversely into twelve 0.5 cm strips

numbered from the origin. Each strip was macerated in 0.02 M sodium phosphate buffer (*p*H 7.35) and incubated with trypsin (200 μg/ml., 37°, 15 min). The kinin released was assayed against synthetic bradykinin.

(b) Immunoelectrophoresis

Fraction C and the kininogen-containing fractions from G-200 Sephadex were concentrated by dialysis followed by freeze-drying. In each case the dried protein from 25 ml. eluate was dissolved in 0.05 ml. 0.10 M veronal-acetate buffer, *p*H 8.6 to give a protein concentration of about 50 mg/ml. These samples were compared with each other and with whole plasma by immunoelectrophoresis using four anti-human sera. Horse anti-human serum, Institut Pasteur, Paris, lot 13461 and lot 223. Horse anti-human serum, Wellcome, London, lot 4505 and lot 4506.

The method of Scheidegger (1955) was followed. Protein samples were placed in circular wells cut in 1% agar gel on microscope slides. A current of 5 mA was passed for one-and-a-quarter hr and antiserum was added to the troughs. The slides were then observed during a period of 72 hr at 4° and photographed under dark ground illumination at intervals between 16 and 72 hr. Ionagar No. 2 (L 12 batch 32) Oxo Ltd, London, was used.

Electrolyte content of solutions

Specific conductivity was measured with a Philips conductivity bridge type PR 9500, at 1,000 c.p.s. Approximate measurements for the location of the electrolyte peak in the eluate from a column were made at room temperature using a micro-cell (constant=0.70).

Accurate measurements were made to check the composition of the buffers used in the stepwise elution of kininogen from DEAE-Sephadex (Table 1). For this purpose the samples were brought to 25±0.5° in a water bath and larger cells were used (constant=1.33).

TABLE 1

THE COMPOSITION OF THE SODIUM PHOSPHATE (0·02 M) BUFFERS USED FOR COLUMN CHROMATOGRAPHY

	NaCl		$NaH_2PO_4.2H_2O$	$Na_2HPO_4.12H_2O$	*p*H	Specific conductivity (25°)
	M	g/l	g/l	g/l		m.mho/cm
A	0·10	5·85	0·50	6·02	7·35	12·2
B	0·13	7·60	0·50	6·02	7·30	14·8
C	0·16	9·35	0·50	6·02	7·25	17·2
D	0·18	10·52	0·50	6·02	7·20	18·8

RESULTS

The purification of human plasma bradykininogen

Step 1 : The preparation of plasma for ion-exchange chromatography

Freshly separated plasma was equilibrated with phosphate buffer by gel-filtration. A volume of 10 ml. was layered beneath buffer on to the top of a gel column (coarse G-50 Sephadex, 2.4 × 15 cm) equilibrated with buffer A (Table 1) at 3 to 5°. The column was immediately developed with the same buffer at a pressure of 20 to 30 cm and a flow rate of 90 ml./hr. The plasma proteins and the bradykininogen were eluted in the first 50 ml. eluate and transferred directly to Step 2.

Fig. 1 illustrates an experiment in which the sodium chloride concentration of the plasma had been increased ten-fold in order to show that the separation of protein from salt was adequate despite the rapid flow rate. The protein peak was contained in the first 50 ml. eluate and separated from the salt peak by about 7.5 ml.

In one experiment the amount of bradykininogen in the protein peak was determined after ethanol precipitation. The applied plasma contained 118 units and the first 50 ml. eluate contained 98 units (83%). The loss of kininogen was explained by the appearance of free kinin in the ethanol solution and in the fractions corresponding with the salt peak.

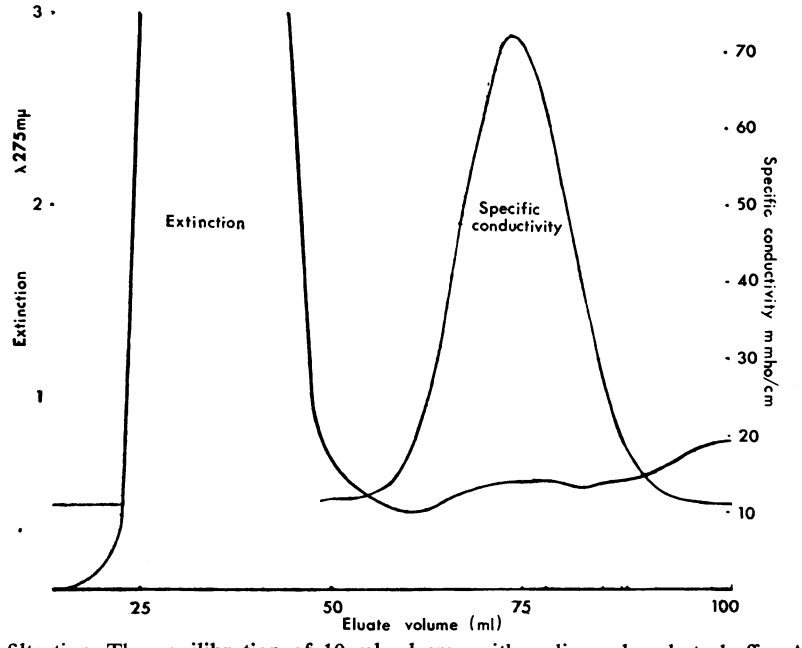


Fig. 1. Gel-filtration. The equilibration of 10 ml. plasma with sodium phosphate buffer A (Table 1) on a column (2.4×15 cm) of G-50 Sephadex. Sodium chloride was added to the plasma in order to demonstrate the separation of protein from salt.

Step 2: Ion-exchange chromatography on DEAE-Sephadex

The eluate from the G-50 Sephadex column was led directly on to a column of DEAE-Sephadex, A-50 (3.5 × 15 cm; dry weight 11.4 g) which had been equilibrated with buffer A. Both columns were united through a ground glass junction and the G-50 Sephadex column was removed immediately 50 ml. eluate had been applied to the lower column. By this means approximately 80% of the kininogen in 10 ml. plasma was applied to the DEAE-Sephadex column and the electrolytes of plasma were not allowed to interfere with adsorption.

The plasma proteins were eluted from the DEAE-Sephadex in four main fractions, A (600 ml.), B, C and D (each 400 ml.) by successive increases in the sodium chloride concentration of the buffer (Table 1). Two measurements were made on aliquots from each fraction:

(i) the kinin (μg Bk) present after incubation for 5 min at 37°.

(ii) the kinin (μg Bk) present after incubation in the presence of trypsin (60 μg/ml.).

Kinin release by trypsin under these conditions was complete in less than 5 min.

In fraction A (i) and (ii) did not differ significantly. Free kinin was present but no kininogen. The free kinin (i) in fractions B, C and D was below the threshold for the

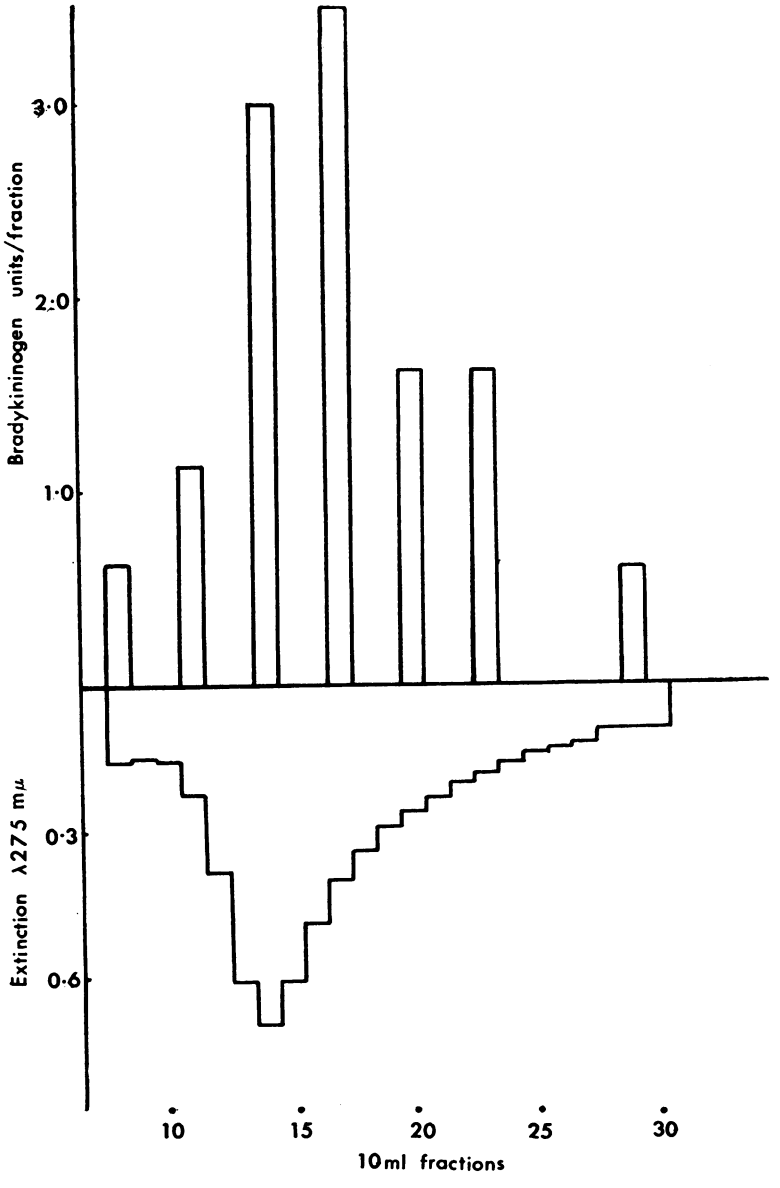


Fig. 2. Anion exchange chromatography. The elution of bradykininogen from a column (3.5×15 cm) of DEAE-Sephadex A-50 by the sodium phosphate buffer (C Table 1). The peak in biological activity coincides approximately with the extinction peak. The unit of bradykininogen is the quantity which in the presence of trypsin will release kinin equivalent to 1 μg synthetic bradykinin.

biological assay. Bradykininogen was present in each of these fractions but notably in fraction C. The results of four experiments with plasma from different donors are summarized in Table 2.

TABLE 2

THE FRACTIONATION OF 10 ML. OF PLASMA ON DEAE-SEPHADEX A-50 (3·5×15 cm)
The first column (i) represents the free kinin present in each fraction. The second column (ii) represents the total kinin present after incubation with trypsin. Each is expressed as μg bradykinin ± Standard Deviation

Fraction	(i)	(ii)
A	2·0±0·7	1·8±0·8
B	<1·6	8·4±4·7
C	<1·0	47·0±9·3
D	<1·0	4·3±1·4

In later experiments fraction C was collected in 10 ml. sub-fractions. Fig. 2 shows the distribution of bradykininogen and extinction (λ275 mμ) values in these fractions. The twenty 10 ml. fractions corresponding with the extinction peak were pooled and freeze-dried in four 50 ml. parts, which provided convenient loads for the G-200 Sephadex columns.

Step 3 : Gel-filtration on G-200 Sephadex

The bradykininogen-enriched fraction C from the DEAE-Sephadex column was fractionated further by gel-filtration on G-200 Sephadex.

Fig. 3 shows the elution pattern of a gel column equilibrated with buffer A and loaded with the freeze-dried protein and salt from 50 ml. fraction C dissolved in 5 ml. distilled water. The column was developed with the phosphate buffer at a hydrostatic pressure of between 1 and 2 m arranged to give a flow rate of 7.5 ml./hour. Fractions of 2.5 ml. were collected and the extinction (λ 275 mμ) of each measured. The specific conductivity

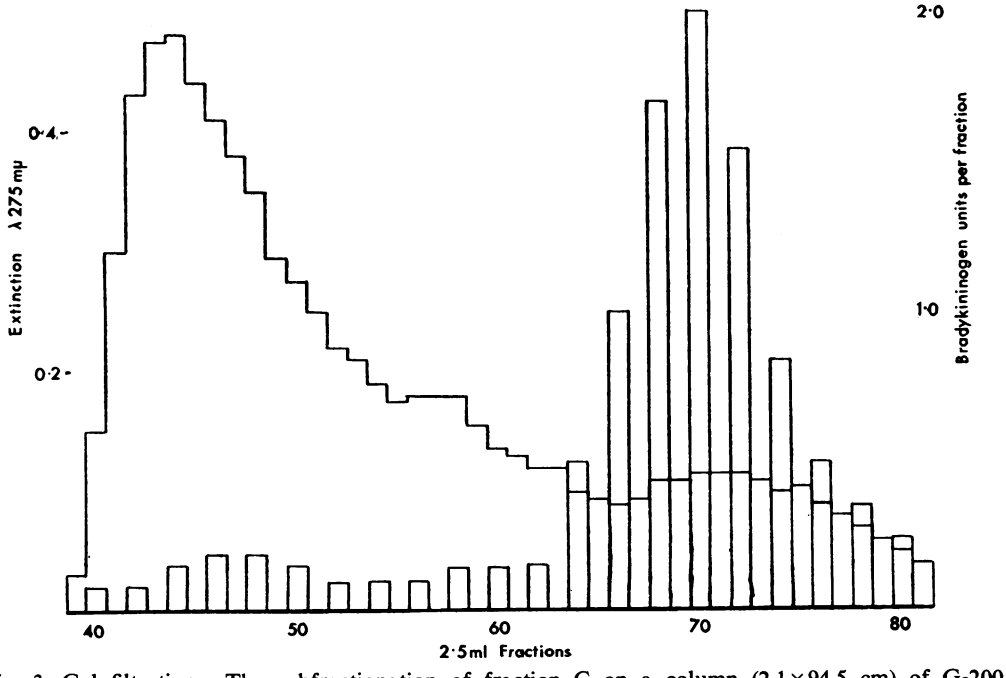


Fig. 3. Gel filtration. The subfractionation of fraction C on a column (2.1×94.5 cm) of G-200 Sephadex in sodium phosphate buffer A (Table 1). Three extinction peaks were observed. The main part of the bradykininogen was eluted with the third extinction peak.

was measured in fractions 92 to 133. Alternate fractions were taken for the assay of free kinin and bradykininogen. Tubes from the first half of the salt peak were assayed for free kinin.

Fractions 40 to 54 contained the main extinction peak. A second flattened peak was detected in fractions 54 to 66 and a third low peak in fractions 66 to 80. Bradykininogen was eluted in all the fractions between 40 and 80 but the activity was concentrated mainly in fractions 64 to 76. A small increase in bradykininogen was detected in fractions 44 to 50. No free kinin was detected in any fraction (limit of detection 0.01 μg/ml.).

Thus fractions 64 to 76 provided a highly purified preparation of bradykininogen obtained from fresh plasma in three steps. Table 3 shows the degree of purification and the loss of bradykininogen which resulted from each step.

TABLE 3

THE PREPARATION OF A PURIFIED BRADYKININOGEN FROM 10 ML. HUMAN PLASMA

	Bradykininogen		
Material	units	%	μg Bk/mg protein
10 ml. plasma	118	100	0·17
Step			
1. Eluate from G-50 Sephadex	98	83	
2. Eluate from DEAE Sephadex			
fractions B, C and D	69	58	0·35
fraction C	60	50	1·3
3. Eluate from G-200 Sephadex			
(third extinction peak)	4×12	40	5·2

The pharmacological properties of purified human bradykininogen

The third step in purification achieved a fourfold increase in specific biological activity. There was no evidence of any other change in pharmacological properties however. Both fraction C and the more highly purified protein from G-200 Sephadex appeared to be equally suitable for most pharmacological purposes. The properties of the two are therefore described together.

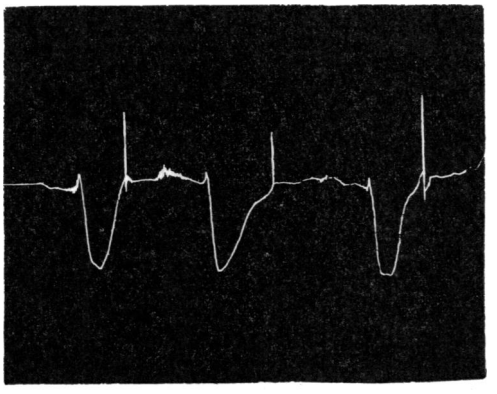
1. *Specific biological activity*

The protein concentrations of fractions 69 and 71 (Fig. 3) were determined by the modified Folin and Ciocalteau method using bovine serum albumin as standard. The potential bradykinin was determined after incubation with trypsin (10 μg/ml.) for 5 min. The kinin was assayed against synthetic bradykinin on the rat uterus using a 2+1 dose design.

Fraction	*Specific activity* (μg Bk/mg protein) (mean ± fiducial limits, P=0.05)
69	6.15 ± 0.90
71	4.89 ± 0.53

The biological activity released by trypsin from fraction C and from the purified kininogen eluted from G-200 Sephadex was bradykinin-like in all the properties studied. The rat uterus, the guinea-pig ileum and the rat duodenum gave qualitatively similar responses to synthetic bradykinin and to the product of kininogen and trypsin. The

kinin released by trypsin from a sample of purified bradykininogen (G-200 Sephadex) was equivalent to 0.8–1.0 μg Bk/ml. when assayed on the rat uterus and equivalent to 0.8 μg Bk/ml. when assayed on the rat duodenum Fig. 4). The kinin was not characterized by chromatography or electrophoresis.



O ● O

Fig. 4. Rat duodenum. The assay of the kinin released by trypsin from purified bradykininogen eluted from G-200 Sephadex. O, 20 ng synthetic bradykinin. ●, the kinin released by trypsin. The dose was equivalent to 20 to 25 ng synthetic bradykinin when assayed on the rat uterus.

2. *Freedom from kininase*

Samples of fraction C from two experiments were incubated with synthetic bradykinin. After 2 hr at 35° the concentration of bradykinin was compared with that of a solution of bradykinin in saline which had received the same treatment and another solution which had been stored at 0°. The results (Table 4) showed no evidence of kininase activity.

TABLE 4

THE STABILITY OF SYNTHETIC BRADYKININ IN FRACTION C

Temperature	Medium	Bradykinin concentration (ng/ml. at 2 h)
0°	0·9% NaCl	12·5
35°	0·9% NaCl	11·1
35°	fraction C	11·7
35°	fraction C	11·1

When the incubation of fraction C (600 μg protein/ml.) with trypsin (10 μg/ml.) was prolonged from 5 to 60 min, there was no destruction of the kinin released. Similarly there was no loss of bradykinin-like activity when a mixture of fraction C and parotid saliva was incubated for a further 55 min. It was not considered necessary to repeat these tests with the more purified bradykininogen from G-200 Sephadex.

3. *Freedom from plasma kinin*

The samples of purified kininogen eluted from DEAE-Sephadex and from G-200 Sephadex were assayed for free kinin in every experiment. The level was invariably

below the threshold for the biological assay ; the rat uterus showed no response either during contact with the sample or during the wash which followed (Fig. 5). In a series of experiments the free kinin in fraction C was less than 1.0 μg Bk whilst the kinin released by trypsin was equivalent to 47 ± 9.3 μg Bk (Table 2).

4. *Freedom from kallikrein*

The absence of kinin-forming enzymes from the preparations was inferred from the following observations.

A solution of fraction C which was capable of releasing 630 ng Bk/ml. within 5 min of the addition of trypsin (10 μg/ml.) contained no detectable kinin (less than 17 ng Bk/ml.) after 30 min at 37° in the absence of trypsin.

Purified bradykininogen from G-200 Sephadex was exposed to acetone (20%, v/v) for 4 hr at 18° as described by Moriya *et al.* (1963); the acetone was then removed by freeze-drying and the product was incubated with fraction C for 5 min at 37°. No kinin was released (less than 5 ng/ml.). Under the same conditions parotid saliva (diluted tenfold) released 130 ng Bk/ml. in less than one min.

Fraction C has been stored as a shell-frozen solution at $-25°$ for eighteen months without the appearance of free kinin on thawing.

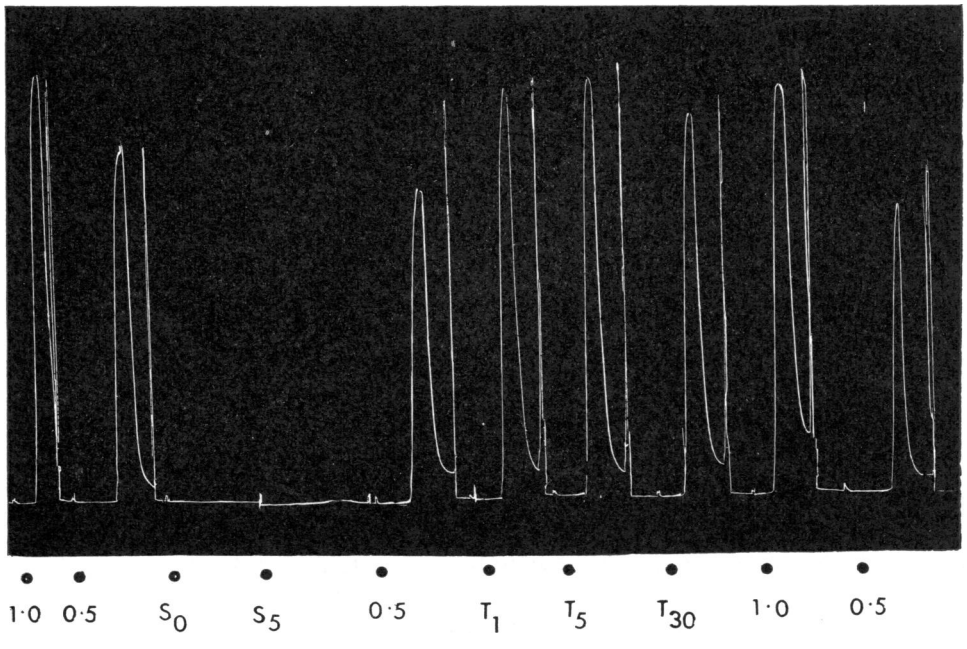


Fig. 5. Rat uterus. The assay of the kinin released by trypsin (60 μg/ml.) from the protein of fraction C (600 μg/ml.) at 37°. 0.5 and 1.0 were ng quantities of synthetic bradykinin. S_0 and S_5 were 0.2 ml. doses of undiluted fraction C. S_5 had been incubated for 5 min. T_1, T_5 and T_{30} were samples of fraction C incubated with trypsin for 1, 5 and 30 min. The incubation mixture was boiled and diluted twenty-fold before 0.1 ml. was applied to the bath.

5. *The action of kinin-forming enzymes*

Trypsin

A sample of fraction C with protein concentration of about 600 μg/ml. was incubated with trypsin (10 μg/ml.). Aliquots were transferred to a boiling water bath at 1, 2, 5 and 30 min. After heating for 10 min they were cooled and the kinin released was assayed against synthetic bradykinin. Maximum kinin release was achieved in 2 to 5 min (Table 5). Similar results are shown in Fig. 5.

TABLE 5

THE RELEASE OF KININ FROM FRACTION C BY KININ FORMING ENZYMES

The kinin was assayed against synthetic bradykinin on the rat uterus. The kinins released by the different enzymes were not characterised

		Kinin concentration ng Bk/ml.			
		Duration of incubation (min)			
Enzyme	Concentration	1	2	5	30
Trypsin	10 μg/ml.	200	220	240	240
Parotid saliva	Diluted× 100	60	80	190	240
	Diluted× 50	110	165	240	
Urinary kallikrein	0·010 Frey units/ml.	10	18	26	135
	0·015 Frey units/ml.	20	20	60	230
Pancreatic kallikrein	0·010 Frey units/ml.	8	11	37	200
	0·015 Frey units/ml.	12	17	40	215
Substrate blank					<2

Parotid Saliva

Saliva from the human parotid duct diluted fifty-fold gave similar results. Full kinin release occurred in less than 5 min.

Purified kallikreins

Preparations of purified kallikrein from hog pancreas and human urine (Moriya *et al*, 1963) released kinin from fraction C. The rate of kinin release was similar when the kallikreins from the two species were present in equal amounts in terms of their declared activity (Frey units).

The physical properties of purified bradykininogen

Starch gel electrophoresis

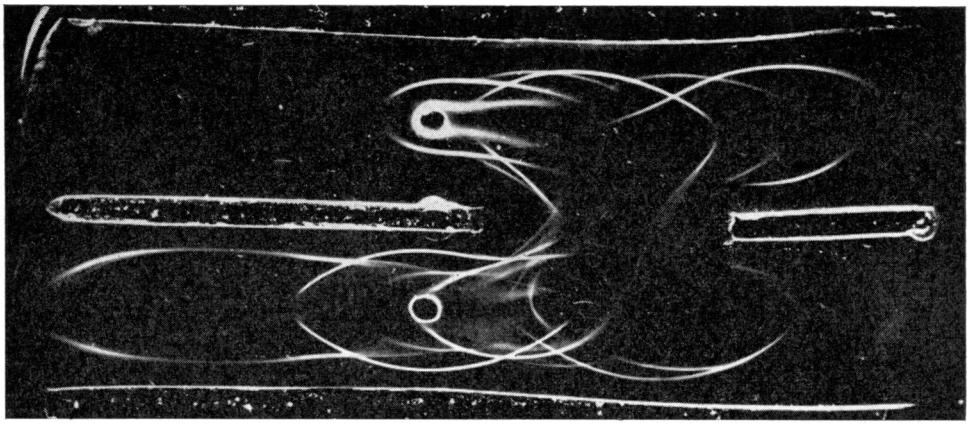
Fraction C was resolved into six bands during electrophoresis in starch gel. These occupied the positions of pre-albumin, albumin, post-albumin, $F\alpha_2$ globulin, $S\alpha_2$ globulin and β lipoprotein (Smithies, 1959). When gels were cut into strips and incubated with trypsin, bradykinin-like activity was detected in the $F\alpha_2$ region (Table 6).

TABLE 6

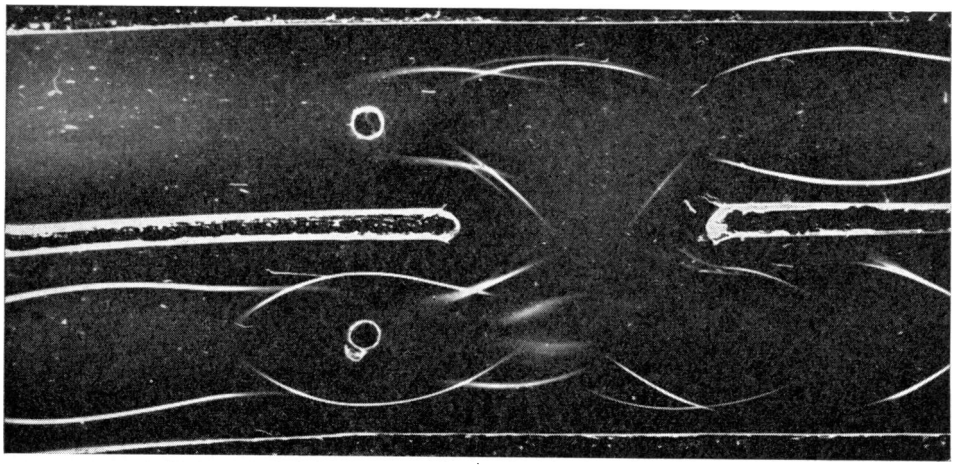
THE BEHAVIOUR OF FRACTION C DURING ELECTROPHORESIS IN STARCH GEL

Strip (× ½ cm. from origin)	Bradykininogen (m-u./strip)	Stained zones (protein/description)
1	less than 3·75	—
2	,,	β lipoprotein
3	,,	$S\alpha_2$
4	,,	—
5	,,	—
6	,,	—
7	5·7	—
8	11·4	$F\alpha_2$
9	5·7	post-albumin
10	less than 3·75	—
11	,,	albumin
12	,,	pre-albumin

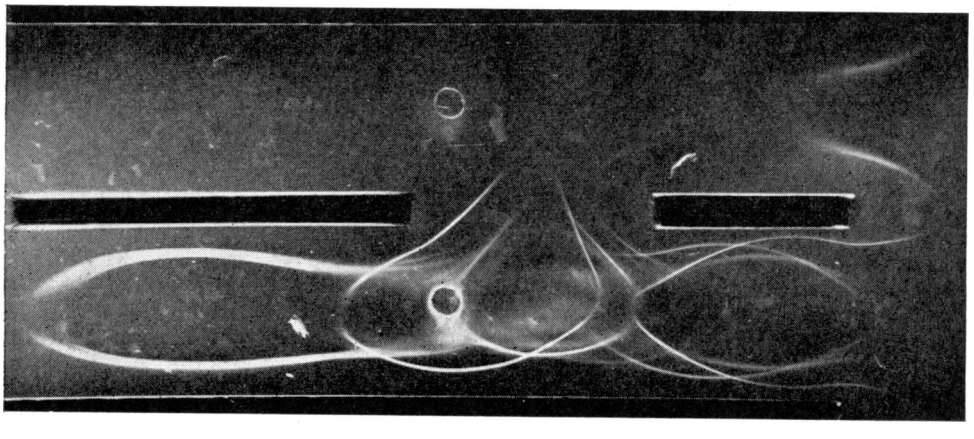
6 (a)



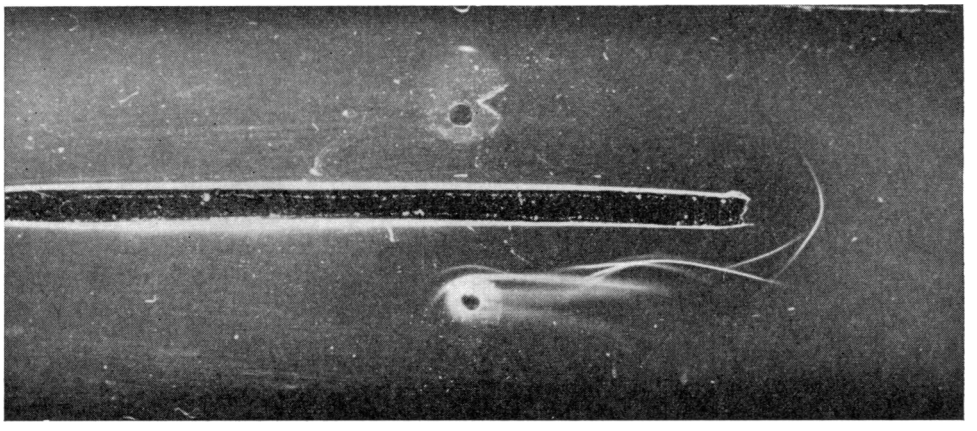
6 (b)



6 (c)



6 (d)



6 (e)

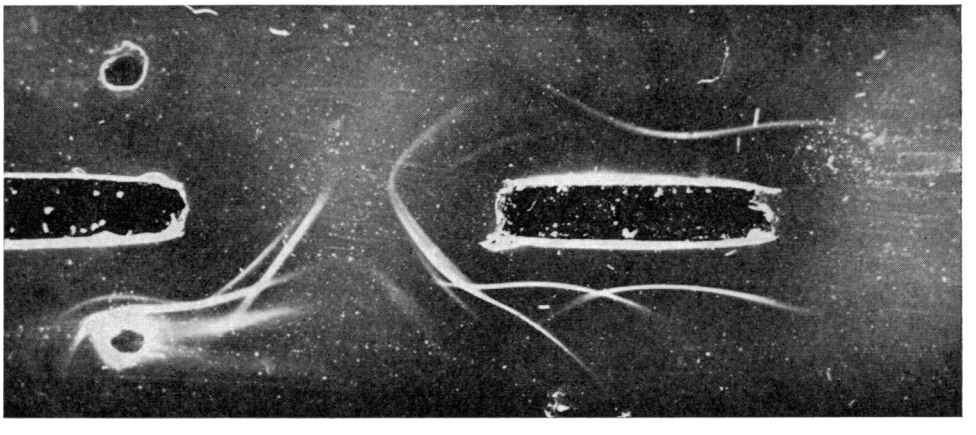


Fig. 6. Micro-immunoelectrophoresis. Partially purified samples of human bradykininogen were compared with each other and with plasma. In each of the experiments illustrated the precipitin arcs were demonstrated using the horse antihuman serum Pasteur 223. The other antisera listed in the Methods section gave fewer arcs. The illustrations are arranged with the origin at the centre, the anode to the right and the more highly purified preparation in the upper half. (a) Compares fraction C with plasma. Eight precipitin arcs were developed by the former. (b) Compares the pooled bradykinin-rich fractions from a G-200 Sephadex column with plasma. The number of precipitin arcs had been reduced to four by the gel-filtration. (c) Shows the fastest component of the more highly purified bradykininogen linking with the albumin of plasma. (d) Shows a similar link with the albumin component of fraction C. (e) Shows a link between the slowest component of a highly purified bradykininogen and a component of fraction C. The purified bradykininogen used in this experiment was eluted from a G-200 Sephadex column (4.0×50 cm). It did not contain the protein corresponding with the precipitin arc close to the origin in (b).

Micro-immunoelectrophoresis

A micromethod for immunoelectrophoresis in agar gel (Scheidegger, 1955) was used to investigate the heterogeneity of the purified bradykininogen.

Fig. 6,*a* compares fraction C with plasma. Eight precipitation arcs are visible. The fastest component in fraction C appears to be albumin. The components which travel slowly during electrophoresis and do not diffuse out from the longitudinal axis probably correspond with the $S\alpha_2$ and β lipoprotein zones on starch gel electrophoresis.

Fig. 6,*b* compares purified bradykininogen from G-200 Sephadex (2.1 × 94.5 cm: combined fractions 63, 65, 67, 73, 75 and 77: Fig. 3) with plasma. Four components are visible. In Fig. 6,*c* the fastest of these is seen to fuse with albumin component of plasma. A similar link is seen in Fig. 6,*d* which compares the purified bradykininogen with fraction C. Fig. 6,*e* shows three components in the purified fraction, the slowest linking with an arc in fraction C.

Gel-filtration

Rabbit γ-globulin, human kininogen, bovine serum albumin and crystalline ovalbumin were compared during gel-filtration on a column of G-200 Sephadex (2.1 × 42.5 cm) equilibrated with 0.50 M NaCl buffered at *p*H 7.1. In each experiment the protein

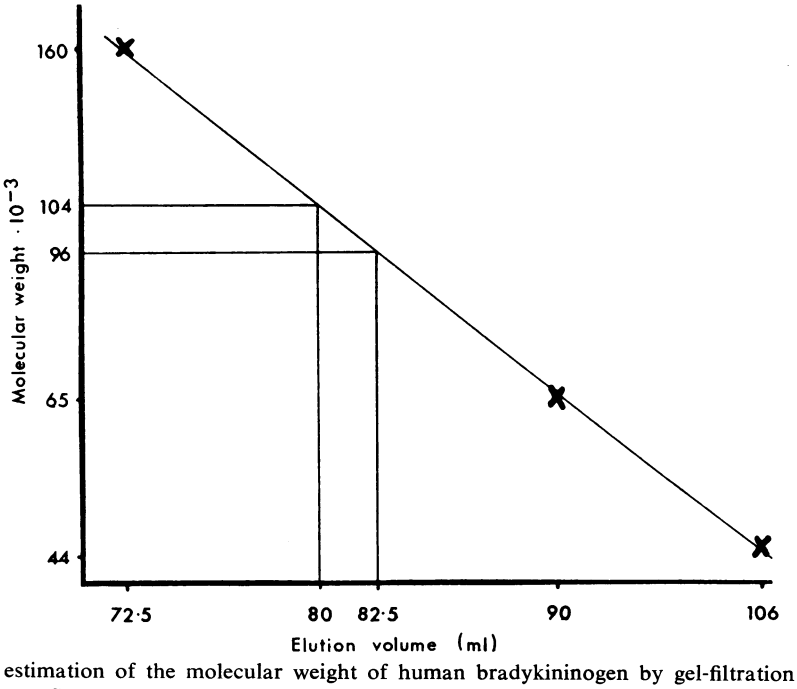


Fig. 7. The estimation of the molecular weight of human bradykininogen by gel-filtration on G-200 Sephadex. Samples of bradykininogen from two donors were compared with rabbit gamma globulin (160,000), bovine serum albumin (65,000) and crystalline ovalbumin (44,000). The elution volumes and the molecular weights were plotted on log scales. The elution volumes for the preparations of human bradykininogen corresponded with molecular weights of 96,000 and 104,000.

(5 to 12 mg) was dissolved in 5.0 ml. buffer which was at least 1.0 M with respect to NaCl, and layered on to the top of the column. The positions of the extinction peak (λ 275 mμ) (or the biological activity peak in the case of kininogen) and the specific conductivity peak were determined. Table 7 gives the volumes of buffer required for the elution of half the protein peak and half the conductivity peak. The variability of the latter is a measure of the failure to achieve complete standardization of the conditions. The values for the molecular weight of the reference proteins are taken from Edsall (1953).

TABLE 7

The elution of human bradykininogen and proteins of known molecular weight from G-200 Sephadex (2·1 × 42·5 cm) in 0·02 M sodium phosphate buffer, *p*H 7·1 containing sodium chloride (0·5 M)

Protein	Elution volume (ml.) Protein	Salt
Rabbit γ globulin (160,000)	72·5	155
Kininogen (donor 1)	82·5	151
Kininogen (donor 2)	80·0	148
Bovine albumin (65,000)	90·5	155
Ovalbumin (44,000)	106·0	159

Fig. 7 shows the curve obtained by plotting log elution volume against log molecular weight (Andrews & Folley, 1963; Whitaker, 1963). The elution volumes for bradykininogen from donors 1 and 2 correspond with molecular weights of 104,000 and 96,000. (Donor 2 was of haptoglobin type 2-2).

DISCUSSION

Development of a suitable purification procedure

The protein precursor of bradykinin can only be detected in retrospect after the peptide has been released by a suitable enzyme. Trypsin was chosen for this purpose, since it was known to release bradykinin from pseudoglobulin, methionyllysylbradykinin and lysylbradykinin. It was assumed that any protein precursor of the three characterized kinins would give bradykinin on incubation with trypsin. The precursors of kinins E, F and S (Armstrong & Mills, 1963) may exist as distinct proteins which do not yield their kinins with trypsin. If this is the case the present investigation is not relevant to these kininogens.

The kinin-forming pro-enzymes of plasma remain inactive under a very narrow range of physical conditions. Immediately a fractionation procedure is started, the plasma bradykininogen is exposed to kinin-forming enzymes and it suffers progressive depletion by kinin release.

Plasma kallikrein is inhibited by dyflos (DFP), soy bean trypsin inhibitor and pancreatic trypsin inhibitor and these substances might therefore be used to protect kininogen during the early stages of purification.

DFP treatment permanently inactivates kallikrein, but it does not prevent the activation of kallikreinogen after the DFP has been removed. It would therefore be necessary to activate all the proenzyme in the presence of DFP, and since inactivation is not immediate, the loss of kininogen at this stage might be considerable. Furthermore the high concentration of DFP which would be required might alter the properties of the kininogen, and change its vulnerability to kallikrein. Since the main object of this

study was the preparation and identification of a physiological substrate for kinin-forming enzymes, any avoidable alteration in the protein molecule was unacceptable.

The trypsin inhibitors from soy bean and pancreas may have been effective in reducing losses of bradykininogen but if any trace had remained in the final product it could have interfered with later enzyme studies.

The established methods for the inactivation of interfering enzymes in plasma (Werle, Götz & Keppler, 1937; Horton, 1959) were deliberately avoided because they probably produce denaturation. There was no advantage in obtaining a high yield of bradykininogen if its characteristics had been altered in the process. It seemed wiser to accept the presence of kinin-forming enzyme during the early stages of the fractionation and to minimize its action by cooling.

An ion-exchange method was used at an early stage in the purification with the hope that it would be possible to separate kinin-forming enzymes from bradykininogen before serious losses had been sustained. After the ionic strength of plasma had been lowered the kinin precursor became tightly adsorbed to DEAE-Sephadex. Large volumes of buffer could be passed through the column and the bulk of the unwanted protein eluted. The kininogen remained firmly bound until the salt concentration of the buffer was increased. When this was done the kininogen was eluted free from kinin-forming enzymes and from kinin. The conditions were adjusted until the best yields were obtained and an attempt was made to increase the scale of the method. When larger volumes of plasma were applied to the column, the yield of kininogen was reduced. This was attributed to the passing of kinin-forming enzymes over the adsorbed kininogen for a longer time.

The combination of steps 1 and 2 (Table 3) gave 50% of the original kininogen in fraction C. One may argue that the half lost was different from the half recovered in being more vulnerable to the kinin-forming enzymes. A recovery experiment with labelled kininogen would be necessary to exclude selective loss. However, we know that small changes in the method reduced the yield sharply. Increasing the length of the G-50 Sephadex column reduced the yield from 50% to 15%, and doubling the plasma load had a similar effect. We can therefore state that the material we normally recover is very readily attacked on the columns, and consider it reasonable to assume that the unavoidable losses occur in this way.

It has been implied above that the losses of kininogen are the result of enzymic action and not caused by the physical conditions used. This assumption is supported by later experience with fraction C. The kininogen in this fraction is very stable. The losses during storage and during prolonged chromatographic separation on G-200 Sephadex are small.

A satisfactory method for the purification of plasma bradykininogen should fulfil the following conditions:

1. The purified protein should be free from kallikrein, kallikrein antagonists, kininase and free kinin.

2. The native state of the protein should be preserved; organic solvents, high temperatures and extreme *p*H levels should be avoided.

3. The protein should retain its biological activity under convenient storage conditions for long periods.

4. The purified protein should be representative of the total bradykininogen present in fresh plasma ; this assumption can only be justified if the method gives a high yield.

5. The quantity of purified kininogen should be adequate for the detailed study of activation by several enzyme systems and for the characterization of the protein.

6. The protein should appear homogeneous during fractionation procedures which offer high resolution.

7. The bradykininogen should have a high specific activity.

The degree to which each of these conditions is satisfied by each of the samples of bradykininogen now available will be discussed.

1. Fraction C and the purified kininogen from G-200 Sephadex are very vulnerable to trypsin and to kallikreins, yet there is no release of kinin when these preparations are incubated alone at 37° for 2 hr. The preparations therefore do not contain kallikrein or trypsin-like enzymes. They could, however, contain an inactive kallikreinogen or a kallikrein which lacked an essential co-factor. Attempts to demonstrate the former by acetone activation failed, but the second possibility is not excluded.

Trypsin and kallikreins release the potential kinin from fraction C and from the kininogen eluted from G-200 Sephadex very rapidly even when the enzyme is highly diluted. This suggests that the preparations are free from enzyme antagonists but there is no quantitative evidence to support this suggestion.

Fraction C does not increase the small loss of bradykinin sustained during 2 hr at 35°. It is kininase-free. No free kinin has been detected in any of the preparations of purified bradykininogen. Purified horse bradykininogen was said to be free from kinin-forming enzyme and assumed to be free from kininase. No supporting data were published however (Henriques, Picarelli & Ferraz de Oliveira, 1962). Purified bovine bradykininogen was free from kinin-forming enzyme but contained kininase (Habermann & Rosenbusch, 1962). The loss of purified human kininogen during dialysis, filtration and freeze drying (Webster & Pierce, 1963) may be due to kinin-forming enzymes.

2. The risk of denaturation during the preparation of fraction C is minimal. The low temperature and the near physiological buffer systems favour the preservation of the native state. The most hazardous step is probably the solution of the freeze-dried protein and buffer salts prior to gel filtration on G-200 Sephadex. The acid treatment of horse plasma (Henriques *et al*, 1962) may cause irreversible changes in the tertiary structure of bradykininogen. The heat coagulation used by Greenbaum & Hosoda (1963) probably denatures bradykininogen.

3. Fraction C has been kept as a shell-frozen solution and as a freeze-dried powder for 18 months without serious loss of activity. The most purified fractions have been stored successfully as freeze dried powders for 12 months.

4. When 10 ml. plasma are passed through steps 1, 2 and 3, about 40% of the original kininogen is obtained in the final product (Table 3). This is not good by absolute standards but it compares favourably with the other methods available for the purification of plasma kininogen. Henriques *et al* (1962) recovered 34% of the kininogen present

in acid-treated plasma but this figure takes no account of the losses which accompanied acid treatment; Greenbaum & Hosoda (1963) recovered less than 3% of the kininogen present in the crude globulin precipitates; Webster & Pierce (1963) obtained 15% of the kininogen present in stored human plasma and Habermann & Rosenbusch (1962) did not quote the final yield.

5. 10 ml. plasma yields about 10 mg protein which contains about 50 units of kininogen. The sensitivity of the rat uterus is so great that this represents about 50,000 detectable doses of bradykinin. The kininogen from 10 ml. plasma is therefore adequate for several activation experiments. The small quantity of protein limits the biochemical investigations which are practicable. Attempts to increase the scale of the method were not successful but several sets of columns were used simultaneously.

6. The most purified bradykininogen is not homogeneous. It contains albumin and at least two α_2 globulins. It is possible that neither of these is the bradykinin precursor. The antisera used in the immunoelectrophoresis were evoked by human sera which probably contained no intact kininogen. The antisera may lack antibodies to human bradykininogen if the loss of kinin alters the antigenicity of the parent molecule. There is little published information about the heterogeneity of other purified kininogens.

7. The specific activity of the most highly purified samples of kininogen is about 6 μg/mg. This compares favourably with the values obtained for other species by other groups. It is inferior however to the values obtained by Habermann & Rosenbusch (1962) for purified ox kininogen. If it is assumed that the molecular weight of the human bradykinin precursor is about 100,000 and that one molecule of bradykinin (mol wt 1131) is released per molecule of precursor, it follows that 6 μg bradykinin are derived from 600 μg protein and that 40% of the purified protein is not kininogen.

The molecular weight of human bradykininogen

During electrophoresis in starch gel the human bradykinin precursor migrated as a fast α_2 globulin. This suggested that its molecular weight was of the same order as caeruloplasmin (mol wt 150,000). The estimates of the molecular weight from the gel filtration experiments were 96,000 and 104,000 (Fig. 7). The behaviour of proteins during gel filtration is however influenced by factors other than molecular weight.

Adsorption of kininogen to G-200 Sephadex would lead to under-estimation of its molecular weight. The relevant experiments were however carried out in 0.5 M NaCl which makes absorption very unlikely. Premature elution leading to exaggeration of the molecular weight could be produced by association with other proteins, deviation from a globular form or by the presence of a large charge on the molecule. The high salt concentration makes association unlikely but the other factors cannot be excluded. The properties of human bradykininogen do not coincide with those of a known α_2 globulin. A detailed comparison will be published separately.

Are there two kininogens in human plasma?

The behaviour of fraction C on G-200 Sephadex is shown in Fig. 3. A small peak in kininogen activity coincides approximately with the first optical density peak. The early kininogen peak is more prominent in columns eluted with buffer of low ionic

strength (0.10 M NaCl) but is still present when 0.5 M NaCl is used. The apparent separation of kininogen into two components may be due to the association of part with a larger protein or to the presence of a second kininogen. The persistence of the early peak in the presence of 0.5 M NaCl suggests that an association phenomenon is not responsible.

Purified bradykininogen as a substrate for kinin-forming enzymes

The study of kinin-forming enzymes under controlled conditions requires a substrate which is free both from kinin and from the plasma enzymes which release and inactivate kinin. For such studies to be physiologically relevant the substrate must resemble closely the kininogen of circulating plasma ; the native state of the protein must be preserved and the yield of kininogen must be high. The method described here satisfies these requirements more closely than do the other methods at present available.

SUMMARY

1. The protein precursor (bradykininogen) of a bradykinin-like polypeptide has been obtained in relatively high yield (50%) from fresh human plasma. The method involves gel-filtration on G-50 Sephadex and ion-exchange chromatography on DEAE-Sephadex.

2. The purified protein does not contain free plasma kinin, kallikrein or kininase and is stable as a freeze-dried powder at 4° and as a frozen solution at −25° for more than one year. The potential plasma kinin is released rapidly by low concentrations of trypsin human parotid saliva, purified urinary kallikrein and hog pancreatic kallikrein. The parent protein migrates as $F\alpha_2$ globulin during electrophoresis in starch gel.

3. Further purification has been achieved by gel filtration on G-200 Sephadex. The product has a specific activity of 5.2 μg bradykinin/mg protein. It gives precipitation lines corresponding to albumin and three other components after immunoelectrophoresis in agar gel. The bradykininogen has a molecular weight of about 100,000.

The authors wish to acknowledge the generous gift of samples of synthetic bradykinin from Dr H. Holgate of Sandoz Products Ltd, and purified kallikreins from Dr E. W. Horton. Mr K. K. Adjepon-Yamoah, Mr A. M. Davison and Mr I. J. Zeitlin assisted during the tenure of undergraduate research scholarships. The work was supported by a grant from the Scottish Hospitals Endowment Research Trust and formed part of the thesis submitted by G. E. Mawer for the degree of Ph.D. at Edinburgh University.

REFERENCES

ANDREWS, P. & FOLLEY, S. J. (1963). Molecular weights of bovine, ovine and porcine pituitary growth hormones estimated by gel filtration on Sephadex. *Biochem. J.*, **87**, 3P.

ARMSTRONG, D. A. & MILLS, G. L. (1963). Chemical characterization of kinins of human plasma. *Nature (Lond.)*, **197**, 490.

CRUFT, H. J. & LEAVER, J. L. (1961). Isolation of histones (?) from Staphylococcus aureus. *Nature (Lond.)*, **192**, 556–557.

CURBY, W. A. (1953). Device for collection of human parotid saliva. *J. Lab. Clin. Med.*, **41**, 493–496.

EDSALL, J. T. (1953). The size, shape and hydration of protein molecules. In: "The Proteins." Vol. I. Part B, 549–716. Academic Press N.Y.

FLODIN, P. (1962). "Dextran gels and their application in gel filtration." p. 43. Meijels Bokindustrie, Halmstad.

GREENBAUM, L. M. & HOSODA, T. (1963). Studies on the isolation of bradykininogen. *Biochem. Pharmac.*, **12**, 325–330.

HABERMANN, E. & ROSENBUSCH, G. (1962). Reinigung und Spaltung von Kininogen aus Rinderblut. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **243**, 357–358.

HENRIQUES, O. B., PICARELLI, Z.P. & FERRAZ DE OLIVEIRA, M. C. (1962). Partial purification of the plasma substrate for the bradykinin-releasing enzyme from the venom of Bothrops jararaca. *Biochem. Pharmac.*, **11**, 707–714.

HORTON, E. W. (1959). The estimation of urinary kallikrein. *J. Physiol. (Lond.)*, **148**, 267–282.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MORIYA, H., PIERCE, J. V. & WEBSTER, M. E. (1963). Purification and some properties of three kallikreins. *Ann. N.Y. Acad. Sci.*, **104**, (Art 1), 172–185.

SCHEIDEGGER, J. J. (1955). Une micro-méthode de l'immuno-électrophorèse. *Int. Archs Allergy appl. Immun.*, **7**, 103–110.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal adults. *Biochem. J.*, **61**, 629–641.

WEBSTER, M. E. & PIERCE, J. V. (1963). The nature of the kallidins released from human plasma by kallikreins and other enzymes. *Ann. N.Y. Acad. Sci.*, **104**, (Art. 1), 91–105.

WERLE, E., GÖTZ, W. & KEPPLER, A. (1937). Über die Wirkung des Kallikreins auf den isolierten Darm und über eine neue darmkontrahierende Substanz. *Biochem. Z.*, **289**, 217–233.

WHITAKER, J. R. (1963). Determination of molecular weights of proteins by gel filtration on Sephadex *Analyt. Chem.*, **35**, 1950–1953.