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SOME DATA ON TWO PURIFIED KININOGENS FROM HUMAN PLASMA

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Work on the purification of the substrate in plasma for kinin-forming enzymes, kininogen, has been carried out in several laboratories. Habermann (1963) thus purified kininogen from bovine serum and Suzuki, Mizushima, Sato & Iwanaga (1965) purified a kininogen from bovine plasma. Habermann (1965, 1966) also studied the structure of the kinin-yielding polypeptides of his purified bovine kininogen (peptic kinin-yielding polypeptides). Pierce & Webster (1966) isolated two kininogens from human plasma. These kininogens were shown to be glycoproteins with molecular weights of about 50,000, as is also the case with the bovine kininogen (Habermann, 1963).

Jacobsen (1966b) found that two different substrates for plasma kinin-forming enzymes could be separated from plasmas of several mammals. These substrates had different molecular dimensions and they differed in their reaction patterns towards various kinin-forming enzymes. The present paper reports the results of further purification with some characterization of these two substrates. The substrate with the larger molecular dimension has been designated substrate 1, and the other and smaller one has been designated substrate 2. The methods employed have been DEAE anion exchange chromatography, gel filtration, disc gel electrophoresis and sucrose gradient ultracentrifugation.

METHODS

Plasma. Citrated human plasma was obtained by using siliconized equipment throughout, as described by Jacobsen (1966b).

Initial purification procedures for kininogens (anion exchange chromatography and gel filtration). The separation procedures previously described (Jacobsen, 1966a, 1966b) have been modified and expanded, so as to include disc gel electrophoresis (see below). The initial procedure was a separation of fractions containing the two kininogens by chromatography of human citrated plasma on a DEAE anion exchanger. Each of the substrates was subsequently exposed to further purification procedures (see below and Table 1). For initial chromatography, plasma (25 ml.) was mixed with 0.05 M Tris-HCl buffer (pH 8) containing 0.25 M sodium chloride (12.5 ml.) and the mixture applied to a DEAE-Sephadex A 50 (A. B. Pharmacia, Uppsala, Sweden) column (18 × 1.8 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8) containing 0.2 M sodium chloride. Substrate 1 is retained on the column while substrate 2 is eluted with the equilibrating buffer. Substrate 1 could then be eluted with a 0.05 M Tris-HCl buffer (pH 8) containing 0.35 M sodium chloride. The effluent containing substrate 2 was treated with " massive contact exposure " in order to eliminate the plasma kallikrein activity (Jacobsen, 1966b), and then applied to a DEAE-Sephadex A 50 column (10 × 1 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8) containing 0.1 M sodium chloride. Substrate 2 was eluted from this column with a buffer containing 0.25 M sodium chloride. Buffer concentrations in the samples were always adjusted to that of the buffer used for equilibration in the following purification procedure.

For further purification those substrate-containing fractions from these initial procedures which contained substrate corresponding to 100 ng/ml. or more were selected. These fractions were then submitted (4 ml. portions), separately for each substrate, to gel filtration on a Sephadex G 200 (A. B. Pharmacia, Uppsala, Sweden) column (43 × 2.7 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 8) and containing 0.2 M sodium chloride. The fractions obtained with the highest substrate content were then further concentrated by applying them again to a DEAE column (10 × 1 cm) and eluting as described above. This method for concentration was found to give less loss of substrate than freeze-drying or ultrafiltration.

The most concentrated substrate-containing fractions now obtained were then submitted to preparative disc gel electrophoresis on separate polyacrylamide gel columns.

All separation procedures were performed at room temperature (20° C). A fraction collector (Radi Rac Automatic, LKB-Produkter, Stockholm, Sweden) was used to collect fractions of 1.75 ml.

Preparative disc gel electrophoresis on polyacrylamide gel was carried out as described for the Canalco Model 12 System (Canalco Corporation, Bethesda, MD., U.S.A.). Stock-solutions (A), (B), (C), (D), (E), (G), (H), (I), and (J) and gel columns were prepared as described in Canalco Chemical Formulation and Instruction for disc eletrophoresis (1965), standard gel. Stock-solution (F) contained 80% sucrose. Sample gel solution was made of stock-solutions (B), (D) and (F) (1:2:2, v/v) with (B) containing 4 mg Riboflavine per 100 ml. Stacking-gel solution was made of (B), (D), (E), (F) and water (1:2:1:2:2, v/v). Separation gel solution was made of (A), (C), water and (G) (1:2:1:4). For the tubes of this apparatus (7.0 × 1.5 cm) 5 vol. of sample gel solution and 3 vol. of the substrate containing solution were mixed and 3.9 ml. of this mixture was applied to the column. Then 2.7 ml. of stacking gel solution was applied and finally the columns were filled with the separating gel solution. The buffer of reservoirs (H) was a tris-glycine buffer (tris, 3 g; glycine, 14.4 g; water to make 1,000 ml.) with pH 8.3. Electrophoresis was here carried out with a current of 20 mA per column for 125 min. These columns were then cut in order to obtain the various fractions. A modification of the gel column cutter described by Heideman (1964) was used and the slices were made 2 mm long. These slices were transferred to clean tubes and 0.7 ml. of 0.1 M Tris-HCl buffer (pH 8) containing 0.2 M sodium chloride was added to each slice. Tubes were stored at −20° C for some hours. The frozen content was then crushed with a teflon mortar and the tubes kept on a shaking-bath at 37° C for 2 hr. The fluid content was then transferred to clean tubes.

The most concentrated substrate containing fractions obtained with this procedure were then used for further studies of the substrate with disc gel electrophoresis, gel filtration and sucrose gradient ultracentrifugation (see below).

Disc gel electrophoresis on polyacrylamide gel was then carried out with the Canalco Model 12 System with glass tubes of 7.0 × 0.5 cm. Stock-solutions, preparation of gel columns and stainings were performed as described in Canalco Chemical Formulation and Instructions for disc electrophoresis (1965), standard gel, with the modifications mentioned above. Sample gel solution, stacking gel solution and separating gel solution were made as described above. Sample gel solution (5 vol.) and purified substrate preparation (3 vol.) were mixed and 0.3 ml. of this mixture was applied to the column. Then 0.2 ml. of stacking gel solution was applied and finally the columns were filled with the separating gel solution.

Electrophoresis was carried out with a current of 2.5 mA per gel column for 65 min and the buffer the same as described above. Destaining was carried out with a current of 10 mA per gel column. The purified substrate solution to be examined was always applied to at least two equal gel columns, one or more of these being used for staining and one or more for fractionation.

Methods for evaluation of molecular dimensions

A. *Gel filtration.* Preparation of the Sephadex G 200 column and gel filtration for estimation of molecular size was carried out as described by Andrews (1965) with some minor modifications. The batch of Sephadex G 200 which was used had a water regain of 20±2 g/g and was allowed to swell in the equilibrating buffer for five weeks. The column (43 × 2.7 cm) was equilibrated with 0.1 M Tris-HCl buffer (pH 8) containing 0.2 M sodium chloride. The flow rate was 12–16 ml./hr

and the sample volume applied to the column was 2 ml. Void volume (V_o) calculated to be 79 ml. and effective internal volume (V_i) to be 166 ml. (Andrews, 1965). Fractions of 1.75 ml. were collected. Standard proteins and concentrations used in this gel filtration procedure are given in Table 1.

B. *Ultracentrifugation* was performed with an International Model B-35 (International Equipment Comp., Needham Heights, Mass., U.S.A.) preparative ultracentrifuge with a swinging bucket rotor, Type SB-269. Centrifugation was performed through a medium containing a linearly increasing sucrose gradient (5–20%) in 0.05 M Tris-HCl buffer (pH 7.5) as described by Martin & Ames (1961). The linear sucrose gradient was prepared in polypropylene tubes in portions of 12.5 ml. and stored for 18 hr at 5° C. The tubes were then placed at 20° C for 1 hr, and 0.2 ml. of the protein solution was layered on top of this gradient. The distance from rotor centre to gradient meniscus was 64.5 mm, to the middle of the protein layer 63.5 mm and to the bottom of the tube 151 mm. Six tubes were centrifuged in the same experiment at 35,000 rev/min for 18 hr and at 20° C. One protein solution was applied to each tube.

Sampling from the tubes was performed by making a hole in the bottom by a fractionator similar to that described by Martin & Ames (1961). Sixty-four fractions of eight drops each were then collected by using the LKB fraction collector equipped with a drop counter. Proteins and concentrations used are listed in Table 3.

The sucrose gradient was tested by mixing Evans Blue (0.4 mg/10 ml.) with the 20% sucrose solution. Perfect linear plots of absorbancy at 605 mμ against fraction numbers were obtained.

The distance from the middle level of the applied protein layer to the middle layer of the fraction with the highest concentration of the protein, was taken as travelled distance of the protein.

Standard proteins. Alcohol dehydrogenase (2 × crystallized from yeast). Albumin (Ovalbumin 3 × crystalline, salt free). Myoglobin from horse (lyophilized). Human albumin (crystallized). All these preparations were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Bovine serum albumin (Sigma Chemical Comp., St. Louis, Missouri, U.S.A.). Human fibrinogen (A. B. Kabi, Stockholm, Sweden).

Protein content of substrate-containing batches was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard.

Protein content of fractions from gel filtration and ultracentrifugation was determined at 280 mμ in a Zeiss spectrophotometer (Model PMQ II) using micro cuvettes of 0.5 ml. With fractions containing very little protein spectrophotometric readings were sometimes carried out at 210 mμ (Tombs, Souter & Maclagan, 1959).

Plasma kinin activity and *amounts of substrates* were estimated as described by Jacobsen (1966b), using the isolated rat uterus preparation for assay. The kinin content of a test sample was calculated from the response of the uterus to a certain dose and to a dose twice as large of both synthetic bradykinin and of the test aliquot.

Kallikreins. Human saliva and the pseudoglobulin preparation of human plasma (Jacobsen, 1966b) were used.

Bradykinin. Synthetic bradykinin (BRS 640) was obtained from Sandoz A. G., Basel, Switzerland.

RESULTS

Purification of substrates

At each stage of the purification procedure determinations of amount of substrate and of protein content were performed as described in Methods. The results from one experiment are listed in Table 1. The yield of substrate given refers to recovery of substrate applied at each stage. The recovery is not calculated relative to the original amounts found in plasma, since only the most concentrated fractions were used for the various purification steps.

TABLE 1

PURIFICATION OF SUBSTRATES FOR PLASMA KININ-FORMING ENZYMES

Substrate	Stage	Purification procedure	Content of protein (mg/ml.)	Amount of substrate (ng/ml.)	(ng/mg protein)	Degree of purification	Yield of applied substrate (%)
Substrate 1		Plasma	73·5	500	6·8		
	I	DEAE-Sephadex column, 1st time	5·2	1,500	288·0	42·4	70
	II	Gel filtration	0·4	300	750	110·3	80
	III	DEAE-Sephadex column, 2nd time	1·2	1,000	870	127·9	84
	IV	Preparative disc gel electrophoresis	0·15	600	4,000	588·2	66
Substrate 2		Plasma	73·5	2,000	27·2		
	I	DEAE-Sephadex column, 1st time	26·4	500	18·9		59·4
	II	Contact exposure	28·8	500	17·4		52·6
	III	DEAE-Sephadex column, 2nd time	60·0	1,500	25·0		70
	IV	Gel filtration	3·4	400	118	4·3	82
	V	DEAE-Sephadex column, 3rd time	7·9	1,500	188	6·9	73·7
	VI	Preparative disc gel electrophoresis	0·16	600	3,750	137·8	58

Amounts of substrates are expressed as kinin that could be developed from the preparations of substrate 1 and 2 on incubation with pseudoglobulin and human saliva respectively. Kinin activity was estimated on the rat uterus preparation and synthetic bradykinin was used as reference. Protein content was determined as described in Methods. Purification evaluations were based on amounts found in plasma. In the first stage all fractions containing more than 100 ng kinin/ml. were used. From gel filtration and onwards only that (or those) fraction representing maximal substrate concentration was used for further purification. " Yield ", therefore, represents the recovery of substrate actually applied in each purification.

A 588-fold and 137-fold purification was obtained for substrate 1 and 2 respectively. In these purified substrate preparations no plasma kallikreins, kininase or kallikrein inhibitory activity towards plasma kallikrein or human saliva could be detected.

Evaluation of molecular dimensions by gel filtration

The final substrate preparations were submitted to gel filtration for estimation of molecular dimensions as described in Methods. Figure 1 and Figure 2 give the elution patterns as regards the two substrates and the content of protein in the various fractions. Elution volumes for different standard proteins and for the two substrates are given in Table 2. In Figure 3 elution volumes are plotted against log molecular weights, and all standard proteins except fibrinogen conform to the relationship as observed by Andrews (1965). The molecular weights of the two substrates, when estimated from this curve according to their elution volume (Andrews, 1965), were found to be about 270.000 and 86.000 for substrate 1 and 2 respectively.

It is claimed that the elution volumes of different proteins from gel filtration are better correlated to the Stokes radius of these molecules than to their molecular weight (Ackers, 1964; Laurent & Killander, 1964; Siegel & Monty, 1965). The Stokes radius and the sedimentation coefficients of the substrates were therefore determined and the molecular weights calculated from these data as suggested by Siegel & Monty (1965).

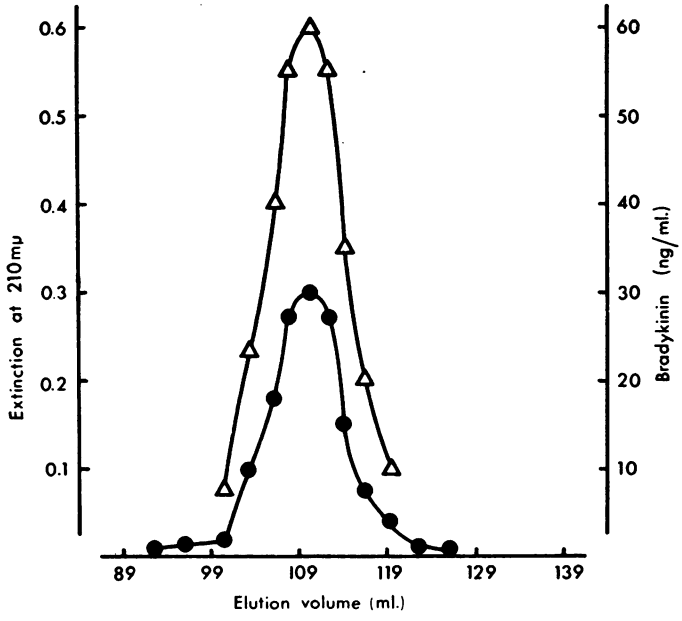

Fig. 1. Elution pattern of final substrate 1 preparation from a Sephadex G 200 column (see Methods). Δ——Δ, content of substrate in the fractions, expressed as ng/ml. of kinin that could be developed on addition of plasma kallikrein, synthetic bradykinin being used as reference (Jacobsen, 1966b). ●——●, content of protein in the fractions, expressed as extinction at 210 mμ (see Methods, and Tombs, Souter & Maclagan, 1959). In the fractions where no substrate content is given, significant kinin development could not be recorded on kallikrein addition.

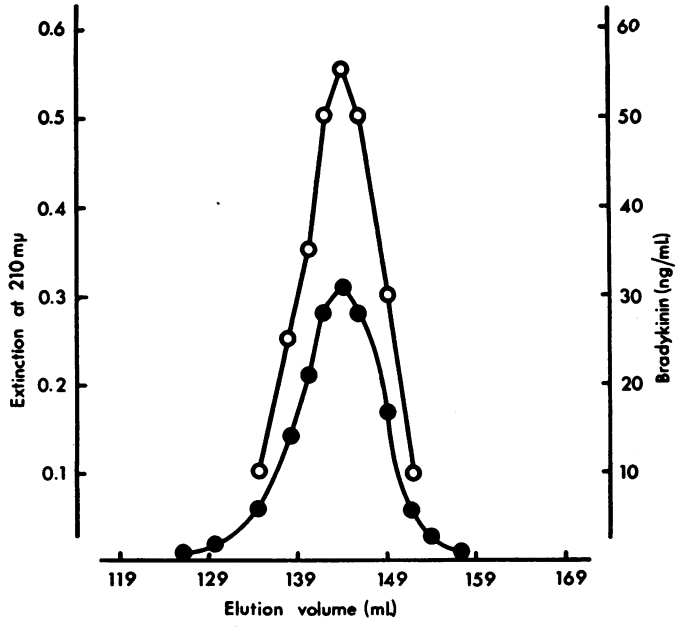

Fig. 2. Elution pattern of final substrate 2 preparation from a Sephadex G 200 column (see Methods). ○——○, content of substrate in the fractions, expressed as ng/ml. of kinin that could be developed on addition of glandular kallikrein (saliva), synthetic bradykinin being used as reference (Jacobsen, 1966b). ●——●, content of protein in the fractions, expressed as extinction at 210 mμ (see Methods, and Tombs, Souter & Maclagan, 1959). In the fractions where no substrate content is given, significant kinin development could not be recorded on kallikrein addition.

TABLE 2

DETERMINATION OF EFFECTIVE PORE RADIUS OF A SEPHADEX G 200 COLUMN AND OF STOKES RADIUS OF SUBSTRATES 1 AND 2

Proteins	Concentration (mg/ml.)	Mol. wt.	a(mμ)	V_e(ml.)	$\frac{V_e-V_o}{V_i}$	r(mμ)	
Ovalbumin	1·0	45.000[1]	2·73[4]	163·85	0·511	18·8	
Bovine serum albumin	1·0	67·000[2]	3·61[5]	152·0	0·440	20·7	average
Human serum albumin	1·0	69·000[3]	3·62[6]	150·0	0·428	20·2	19·6 mμ
Alcohol dehydrogenase	1·0	150·000[3]	4·55[6]	128·0	0·295	18·6	
Substrate 1	0·15	197·000	6·2	110·0	0·187		
Substrate 2	0·16	57·000	3·8	143·75	0·390		

Each protein solution (2 ml.) was applied to the Sephadex G 200 column (43 × 2·7 cm). The effective pore radius of the column (r) was calculated by the help of the four standard proteins (see text), whereafter the Stokes radius of the two substrates could be evaluated. a: Stokes radius, V_e: elution volume, V_o: void volume (79 ml.), V_i: internal volume of column (166 ml.).

Data for the known standard proteins, see: [1] Gutfreund (1944); [2] Phelps & Putnam (1960); [3] Hayes & Velick (1954); [4] Laurent & Killander (1964); [5] Rogers, Hellerman & Thompson (1965); [6] Ackers (1964).

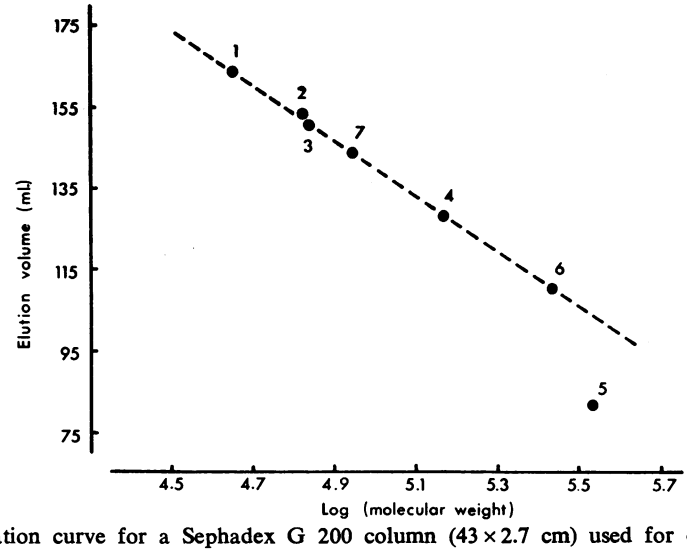

Fig. 3. Calibration curve for a Sephadex G 200 column (43 × 2.7 cm) used for determination of molecular weights according to Andrews (1965). The elution volumes of different proteins (Table 2) are plotted against log molecular weight. The numbers at the plots indicate the following proteins: 1 = ovalbumin; 2 = bovine serum albumin; 3 = human serum albumin; 4 = alcohol dehydrogenase; 5 = human fibrinogen. At 6 and 7 substrate 1 and 2 are plotted according to their elution volume.

A basic equation to be applied for the determination of the Stokes radius of a molecule or the effective pore radius of the gel, when using a molecular sieve column is given by Ackers (1964):

$$\frac{V_e - V_o}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

V_e is the elution volume for a protein, V_o is the void volume and V_i the effective internal volume of the column. The Stokes radius of the molecule is denoted *a* and the effective pore radius of the gel *r*.

This equation is predicated on the assumption that the passage of a molecule through the column can be characterized as a restricted diffusion process dependent upon both the Stokes radius of the effluent molecule and the effective pore radius parameter of the molecular sieve (Ackers, 1964). The pore radius in our gel column can be calculated from this equation using data from gel filtration of macromolecules with known Stokes radii as given in Table 2. With the effective pore radius known, the equation can then be used for determination of Stokes radii of unknown proteins. Siegel & Monty (1965) found an excellent correlation between elution position and Stokes radius with molecules as large as 10.7 mμ.

The average pore radius of this gel was calculated to be 19.6 mμ. The Stokes radius of substrate 1 and 2 could then be calculated to be 6.2 and 3.8 mμ respectively based on their elution volumes and the column parameters V_o, V_i and r given in Table 2.

Determination of sedimentation coefficients of the substrates

Ultracentrifugation was carried out as described in Methods. Assuming a partial specific volume of 0.725 cm^3/g of proteins used, the sedimentation coefficients ($S_{20,w}$) were estimated from the following ratio, by using standard proteins with known coefficients as markers (Martin & Ames, 1961):

$$\frac{\text{distance travelled by unknown protein}}{\text{distance travelled by standard protein}} = \frac{S_{20,w} \text{ of unknown protein}}{S_{20,w} \text{ of standard protein}}$$

The calculated values from sedimentation experiments are given in Table 3. Sedimentation coefficients of substrate 1 and 2 were estimated to about 7.68 $S_{20,w}$ and 3.65 $S_{20,w}$ respectively.

TABLE 3

ESTIMATION OF SEDIMENTATION COEFFICIENTS BY ULTRACENTRIFUGATION THROUGH SUCROSE GRADIENT

Reference Protein	Calculated sedimentation coefficients ($S_{20,w}$)	
	Substrate 1	Substrate 2
Bovine serum albumin ($S_{20,w}$ = 4·41[1])	7·67(7·60–7·80)	3·57(3·50–3·65)
Ovalbumin ($S_{20,w}$ = 3·42[2])	7·75(7·70–7·80)	3·67(3·65–3·70)
Myoglobin ($S_{20,w}$ = 2·04[3])	7·53(7·50–7·60)	3·57(3·50–3·60)
Alcohol dehydrogenase ($S_{20,w}$ = 7·6[4])	7·78(7·75–7·80)	3·77(3·70–3·80)

The sedimentation coefficients of the two substrates were estimated by ultracentrifugation at 20° C through sucrose gradient (see Methods and Results), using proteins with known $S_{20,w}$ values as markers. Concentration of standard proteins was 2·5 mg/ml. and of substrate 1 and 2 0·15 and 0·16 mg/ml. respectively. 0·2 ml. of the protein solution was applied to each tube containing 12·5 ml. sucrose gradient solution. Sedimentation coefficients of reference proteins are given in parentheses to the left (refer: [1] Phelps & Putnam (1960); [2] Creeth & Winzor (1962); [3] Edsall (1953); [4] Martin & Ames (1961)). The sedimentation coefficients of the substrates have been calculated relative to those of the various standard proteins as described in Results. Average values for three separate estimations are given (with the spread of the values in parentheses).

Molecular weights of substrates

With the Stokes radius and the sedimentation coefficient known, the molecular weight (M) can be calculated from the following equation (Siegel & Monty, 1965):

$$M = \frac{6 \cdot a. S_{20,w} \cdot N \cdot \pi \cdot \eta}{1 - \bar{v} \cdot \rho}$$

Viscosity of water is denoted by η, density of water at 20° C by ρ, Avogadros number by N and partial specific volume by v, $S_{20,w}$ is the sedimentation coefficient and a the Stokes radius.

The molecular weights of substrate 1 and 2 were thus calculated to be about 197.000 and 57.000 respectively. Equal results were obtained when more crude substrate preparations were submitted to gel filtration and ultracentrifugation.

Disc gel electrophoresis

Purified substrate preparations and whole plasma were examined by this method, and the results from these experiments are illustrated in Fig. 4. Of two equal columns one was fractionated and the other stained. Each substrate could be demonstrated in a single band, different from that of the other substrate, when applied alone or together with the other substrate to the gel columns. Conversion of one substrate to the other was not observed.

The mobility of the various proteins on disc gel electrophoresis (Clarke, 1964) was compared with the mobility of the substrates. Substrate 1 moved like the beta-globulins and substrate 2 like alpha-1-globulins, as is seen from Fig. 4.

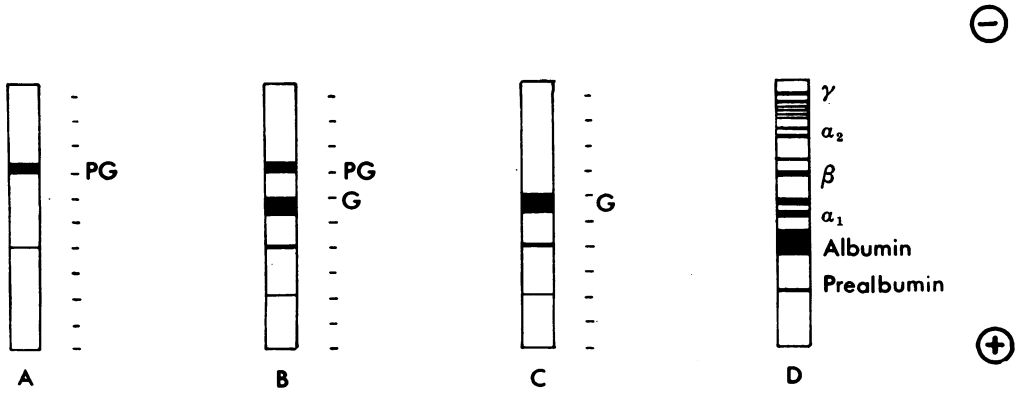

Fig. 4. Patterns given by purified substrates 1 and 2 and by human plasma in stained and fractioned gel columns from disc electrophoresis (see Methods and Results). The stained columns are diagrammatically illustrated. The graded scales denote fractions of 4 mm each from similar columns, where tests on kinin formation were carried out: A=substrate 1; B=mixture of substrate 1 and 2; C=substrate 2; D=human plasma (1:30 dilution). P indicates kinin formation when the eluted fraction was incubated with pseudoglobulin preparation, and G indicates kinin formation on incubation with human saliva. Kinin activity was tested on the isolated rat uterus preparation.

DISCUSSION

The recovery values show that relatively large amounts of the substrates were lost during the purification procedures. This was to be expected during the first stages where plasma kallikrein was still found together with the substrates. Some kallikrein might easily be activated by the various procedures. In the subsequent purification steps some other mechanisms must probably account for the further loss of substrate activity.

The substrate 1 preparation obtained from the preparative disc gel electrophoresis did apparently contain only small amounts of other proteins, and was thus purified to a satisfactory degree. The purification ratio was not higher than 588 though, as judged from the ability to liberate kinin. This relatively low substrate content in the final preparation could be explained if only a minor portion of the large substrate molecule forms active polypeptides. The possibility also exists that denatured substrate molecules are present in the substrate containing band. If such proteins behave similarly to the kinin-yielding substrate throughout the separation procedure, but are unable to release any kinin, an apparently low substrate concentration would be observed.

The purification of substrate 2 was less successful as judged from the results of disc electrophoresis and from the small amount of kinin liberated per mg of protein in the final preparation. This may in part be the result of denaturation caused by the rather rough treatment called " massive contact exposure ".

The solutions of purified substrates 1 and 2 had a much lower protein concentration than of the solutions of standard proteins in the samples submitted to gel filtration and ultracentrifugation. The differences in concentrations were, however, within the limits given by Martin & Ames (1961) and Ackers (1964) for the use of the methods.

For calculation of sedimentation coefficients and molecular weights a partial specific volume of 0.725 cm^3/g of protein has been assumed for each protein. The partial specific volume of most proteins lies between 0.700 and 0.750 cm^3/g (Edsall, 1953). This general assumption will thus result in a small error in the estimation of $S_{20,\,w}$ for most proteins (Martin & Ames, 1961). This error can be of only minor importance in the various calculations.

Substrate 1, with its larger molecular dimensions, could theoretically be a polymer or an aggregate of the smaller substrate 2. The two substrates behaved differently, however, in all separation procedures, and no conversion of one substrate to the other was observed. Not even during disc electrophoresis could any conversion of substrate 1 to substrate 2 be observed, and this procedure is known to have a marked dissociation effect on complexes (Ornstein, 1964). The reaction pattern of the substrates with various kinin-forming enzymes also remained the same after disc gel electrophoresis.

From the purified substrate 1, 4 μg kinin activity was liberated per mg of protein, synthetic bradykinin being used as reference. Webster & Pierce (1963) found that bradykinin was the kinin formed when plasma kallikrein acts on plasma proteins. Bradykinin has a molecular weight of about 1060. If it is assumed that bradykinin is the kinin formed here from substrate 1, and if it is further assumed that one molecule of bradykinin is formed from each molecule of substrate 1, the molecular weight of substrate 1 should be about 265.000. This is the upper limit of the molecular weight for a substrate yielding only one molecule of bradykinin, as the presence of proteins unable to release kinins

would result in lower molecular weight. Such a molecular weight is in accordance with values obtained with the various methods previously described. It could well be, however, that several polypeptides are liberated from each molecule of native substrate. Simultaneously there could be some loss of kinin activity or a substantial portion of the protein could be denatured and unable to release kinin. A formation of kinins other than bradykinin with a different effect on the smooth muscle preparation cannot be excluded.

The molecular weights of the substrates have been calculated from gel filtration data directly as well as from these data combined with findings from sucrose gradient ultracentrifugation. The substrate proteins with smaller molecular dimensions are supposed to be glycoproteins (Habermann, Klett & Rosenbusch, 1963 ; Pierce & Webster, 1966), and this may explain the discrepancies between values for molecular weights obtained by these two methods, as some glycoproteins do not conform to the ordinary relationship for proteins between elution values from gel filtration and log molecular weight (Andrews, 1965 ; Siegel & Monty, 1965).

It seems likely that the purified substrates obtained by other authors may be identical with or similar to substrate 2, but different from the substrate 1 described in this paper. Rosenbusch (1962) did demonstrate two substrates in bovine serum with apparently different molecular dimensions. Habermann (1963), however, found one substrate in bovine serum with molecular weight of 48.000 and with a sedimentation coefficient of 3.58. He also showed that the larger substrate demonstrated by Rosenbusch (1962) was a dimer of the smaller substrate molecule and occurring at low pH. Pierce & Webster (1966) separated two substrates from human plasma, both with molecular weights of about 50.000. They mentioned another minor substrate fraction with an apparently larger molecular dimension, but no further information was given for this fraction.

Purified substrates previously described thus resemble substrate 2 as regards the molecular weight of about 50.000, the sedimentation coefficient and also mobility on electrophoresis (Habermann *et al.*, 1963).

The findings in lymph where the representation of the substrates is different relative to that in plasma (Jacobsen, 1966c ; Jacobsen & Waaler, 1966) strongly support the concept of two substrates with different molecular dimensions. The available data thus indicate that in plasma there exist two different substrate entities, with different molecular dimensions, and that these two substrates are differently attacked by kinin-forming enzymes.

SUMMARY

1. Further work has been carried out on the purification and characterization of two different plasma kininogens, substrate 1 and substrate 2, the separation of which has been described in previous publications. The methods employed have been DEAE anion exchange chromatography, gel filtration, disc gel electrophoresis and sucrose gradient utlracentrifugation.

2. For substrate 1, which yields kinins with plasma kallikrein as well as with glandular kallikrein, a 588-fold purification was obtained. For substrate 2, which reacts with glandular kallikrein, a 137-fold purification was achieved.

3. From the results of gel filtration experiments the Stokes radius was calculated to be 6.2 mμ for substrate 1 and 3.8 mμ for substrate 2. Estimations based on the results from sucrose gradient ultracentrifugation gave sedimentation coefficients ($S_{20,w}$) of 7.68 and 3.65 for substrate 1 and 2 respectively.

4. From the values for Stokes radii and sedimentation coefficients the molecular weights of substrate 1 and substrate 2 were calculated to be 197.000 and 57.000 respectively.

5. Each of the two kininogens moved as one single band on disc gel electrophoresis at pH 8.3. Substrate 1 moved like the beta-globulins, substrate 2 like alpha-1-globulins. Substrate 1 was apparently not a polymer or an aggregate of the smaller substrate 2.

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