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The Sialic Acid Content and Isoelectric Point of Human Kininogen

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The sialic acid content of highly purified human kininogen was found to be about 8.6mol/mol (mol.wt. 50000). The isoelectric point (pH4.9 ± 0.2) is much higher than that of bovine low-molecular-weight kininogen, but is close to that expected from the amino acid and sialic acid analyses.

The kininogens are plasma proteins from which the pharmacologically active peptides kallidin and bradykinin are derived by proteolytic action. Low-molecular-weight kininogens have been purified from several mammalian species. Smaller amounts of higher-molecular-weight forms may occur naturally or be products of polymerization at low pH. These subjects have been reviewed (Habermann, 1970; Pierce, 1968, 1970). The bovine low-molecular-weight kininogen purified by Suzuki and co-workers (Kato *et al.*, 1967) has a molecular weight of 49000 and an apparent biological specific activity of 20μg of bradykinin/mg of protein, which is the theoretical maximum (Habermann, 1963). Complete amino acid and carbohydrate analyses have been reported (Nagasawa *et al.*, 1966) and the isoelectric point was estimated to be pH3.3–3.6 (Habermann, 1963; Habermann *et al.*, 1963; Kato *et al.*, 1967). Two forms of human low-molecular-weight (about 50000) kininogen were purified by Pierce & Webster (1966) and their amino acid composition was similar to that of the bovine kininogen. The sialic acid content and isoelectric point were not determined. However, isoelectric points between pH4.5 and 5.2 have been reported for human kininogens of mol.wt. 70000 but unknown purity (Spragg & Austen, 1971; Spragg *et al.*, 1973; Wuepper & Cochrane, 1971; Habal & Movat, 1972). In view of this apparent discrepancy between the pI of human and bovine kininogens, it seemed highly relevant to determine both the isoelectric point and the sialic acid content of a highly purified human low-molecular-weight kininogen with an amino acid composition similar to those reported by Pierce (1968) for human kininogen and by Nagasawa *et al.* (1966) for bovine kininogen.

Experimental

Kininogen was purified from pooled human plasma by $(NH_4)_2SO_4$ fractionation, gel filtration and DEAE-cellulose chromatography as described elsewhere (Hamberg *et al.*, 1975). The specific activity of the kininogen was more than 14μg of bradykinin/mg of protein in the guinea-pig ileum assay of the peptide released by trypsin (Hamberg, 1969). Polyacrylamide-gel electrophoresis (Davis, 1964) at pH8.9 with 50μg of protein revealed a major impurity with very similar electrophoretic mobility to that of kininogen, and it was immunologically identified as α-2HS glycoprotein (Schultze & Heremans, 1966). By analysis of Partigen plates by the Mancini *et al.* (1965) method the content of α-2HS glycoprotein was 30% by weight.

Other methods. Isoelectric focusing was done on 5% polyacrylamide gels (67mm×3mm) containing 1% (w/v) Ampholine (pH3–6; LKB, Bromma, Sweden), 0.001% riboflavin, 0.05% *NNN'N'*-tetramethylethylenediamine and 5% (v/v) glycerol. The cathode and anode electrolytes were 0.5% ethylenediamine and 0.5% phosphoric acid respectively. During focusing the voltage was allowed to increase to 200V, not exceeding a current of 1mA per gel. The pH was measured of aqueous extracts of slices of representative gels. Gels were stained for protein with Bromophenol Blue (Awdeh, 1969).

For assay of kininogen each gel slice was homogenized with 3.5ml of 50mM-Tris–HCl, pH7.8, dialysed overnight against this buffer, and then incubated for 20min at 37°C with 10μg of trypsin (TRL; Worthington Biochemical Corp., Freehold, N.J., U.S.A.), and filtered through Whatman no. 1 paper. The filter was washed with 1ml of buffer and 2ml of 70% (v/v) ethanol, and 10ml of 96% (v/v) ethanol was added directly to the filtrate. The filtrate was boiled briefly and dried *in vacuo* at 60°C. The residue was dissolved in 1ml of water and assayed on an isolated rat uterus, with synthetic bradykinin triacetate (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as a standard (Hamberg, 1959, 1969).

For immunodiffusion analysis by the method of Ouchterlony (1948) gel slices were macerated in an equal volume of the buffer used in the agar (Purified Agar; Difco, Detroit, Mich., U.S.A.) plates (8.5g of NaCl, 15g of glycine, 8.15g of sodium diethyl-

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barbiturate, 100mg of sodium methiolate per litre of water, pH adjusted to 7.6 with conc. HCl). The slurry was placed in the well directly on Ouchterlony plates. Commercial anti-(α-2HS glycoprotein) serum (Behringwerke A.G., Marburg/Lahn, W. Germany) and an anti-kininogen serum K-1 (Hamberg & Tallberg, 1972) were used. The latter serum contained antibodies primarily against kininogen and also (weaker) against α-2HS glycoprotein. The anti-kininogen titre was higher than 1:8. Protein was determined by assuming $E^{1\%}_{280} = 10.0$ at pH7.8.

Results and discussion

Fig. 1 shows the result of a typical isoelectric-focusing experiment. In the absence of urea (gel *a*) the major protein band focused at pH4.9±0.2. Two or more rather variable minor bands accompanied the main band, and a faint band, apparently α-2HS glycoprotein, was usually detected at pH4.4. In gel (*c*) the kininogen content of slices cut from the gel was determined. The recovery of activity was 32%, and a sharp peak was found corresponding to the main band in gel (*a*). Very little activity was found at pH4.4, and in parallel experiments slices from this region of the gel formed a single precipitin line of identity with anti-(α-2HS glycoprotein) and K-1 sera. In contrast, slices containing the main protein band formed a single precipitin line with K-1 serum but failed to react with anti-(α-2HS glycoprotein) serum. In 4.9M-urea the pH gradient was slightly shifted to the alkaline side. The faint band at pH4.4 could no longer be detected, and a single sharp band was focused at pH5.1 (gel *b*). The disappearance of the satellite bands in the presence of urea suggests that they are probably conformational isomers of the main component.

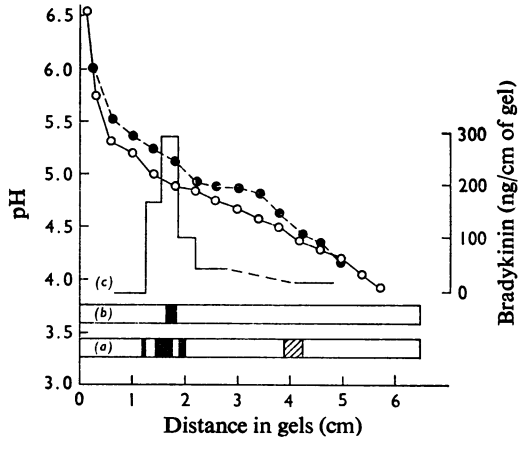

Fig. 1. *Isoelectric focusing of kininogen in the presence and absence of* 4.9M-*urea*

Kininogen was dissolved in the incipient gel mixture, 25μg each in gels (*a*) and (*b*) and 48μg in gel (*c*). Gel (*b*) contained 4.9M-urea. Electrophoresis was carried out at 25°C for a total of 5h. The initial voltage was 26V and the final voltage of 200V was reached after 3h. Gels (*a*) and (*b*) were stained for protein, and slices from gel (*c*) assayed as described in the test (results shown as histogram). The pH gradients determined in blank gels in the presence (●) and absence (○) of urea are shown. Other experimental details are described in the text.

In the experiments of Fig. 1 the kininogen was applied to the gels by including it in the incipient gel mixture before u.v. illumination. The same pattern of protein bands was obtained when kininogen was applied to the alkaline end of the gels as 60μl of a 0.6mg/ml solution in 2% (w/v) sucrose, pH7.5. In this case the kininogen was not exposed to the gelling process, nor to pH values below its isoelectric point, and there is no reason to suspect structural alterations of the type reported to occur with bovine kininogen at pH3.5 (Habermann, 1963). Possible modification of kininogen during the later stages of the purification procedure was investigated by focusing material purified only through the first gel-filtration step, and with a specific activity of about 1μg of bradykinin/mg of protein. After 1.9mg of protein had been focused, the gel was assayed with a rat uterus. The total activity recovery was 19%, and 77% of this was in a slice cut between pH4.3 and 4.9.

It was possible that kininogen exhibited an anomalously high pI in the presence of Ampholines. However, when kininogen dissolved in 120mM-sodium acetate, pH4.1, containing 10% glycerol was applied to the anode and cathode ends respectively of 5% polyacrylamide gels containing 50mM-sodium acetate, pH4.4, protein-staining material was detected moving towards the cathode but not towards the anode, consistent with an isoelectric point higher than pH4.1 in the absence of Ampholine.

The sialic acid content of two samples of kininogen was determined by small-scale modifications of the copper–resorcinol procedure (cf. Spiro, 1966) and the thiobarbituric acid procedure of Aminoff (1961), standardized with *N*-acetylneuraminic acid (Sigma). In the copper–resorcinol test the E_{580}/mg of kininogen was equivalent to 57μg of *N*-acetylneuraminic acid before correction for the small extinction due to neutral sugar, and to 52μg after application of this correction. Before the thiobarbituric acid assay, protein samples were hydrolysed at 86°C in 0.1M-H_2SO_4. Release of sialic acid was maximal after 45min hydrolysis and was 44±6μg/mg of protein. Under these conditions 15% destruction of free *N*-acetylneuraminic acid occurred. Correction for this destruction gives a sialic acid content of 52±7μg/mg of protein. Since the kininogen preparations contained 30% by weight of α-2HS glycoprotein (see

above) with a sialic acid content of 40 μg/mg (Schultze & Heremans, 1966), the sialic acid content of the kininogen alone is about 55 μg/mg (i.e. 8.6 mol/50000g), which is the same as reported for bovine kininogen (Nagasawa *et al.*, 1966).

Despite the close agreement between the sialic acid content of the present human kininogen preparation and of the bovine kininogen of Suzuki's team (Nagasawa *et al.*, 1966), the evidence reported here appears convincing that, in the presence and absence of urea, the human kininogen exhibits a much higher isoelectric point than does the bovine. Our pI of 4.9 ± 0.2 is in agreement with Spragg & Austen's (1971) results with uncharacterized human kininogen. Preliminary amino acid analysis (Hamberg *et al.*, 1975) of the present preparation showed that, both before and after correction for the presence of 30% by weight of α-2HS glycoprotein, the proportion of acid and basic residues was very similar to that of the bovine kininogen, with the important exception that the present preparation contained only 20 mol of amide N instead of 50. In fact, the isoelectric point reported for bovine kininogen appears to be anomalously low. From the data of Nagasawa *et al.* (1966), and assuming the sialic acid residues to be fully ionized and a pK of 4.3 for the side-chain carboxyl groups and 6.5 for histidine, the isoelectric point can be calculated to be at about 6.0 if all ionizable groups titrate normally. Even assuming 20 mol of amide N instead of the 50 reported only decreases the theoretical isoelectric point to pH 5.3. The human kininogen of Pierce & Webster (1966) apparently contains rather fewer side-chain carboxyl groups, but otherwise closely resembles the bovine kininogen in amino acid composition (Pierce, 1968). The observed pI of the present preparation of human kininogen is only slightly less than that (pH 5.3) calculated from its amino acid composition, suggesting that its ionizable residues are exposed and titrate normally. In contrast, the low pI observed for bovine kininogen suggests that this protein may contain basic residues trapped in the uncharged form. Release of these residues from the interior of the molecule and their resultant ionization might account for the irreversible structural alterations occurring to bovine kininogen at pH 3.5 (Habermann, 1963).

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Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
Awdeh, Z. L. (1969) *Sci. Tools* **16**, 42–43
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Habal, F. M. & Movat, H. Z. (1972) *Res. Commun. Chem. Pathol. Pharmacol.* **4**, 477–486
Habermann, E. (1963) *Biochem. Z.* **337**, 440–448
Habermann, E. (1970) in *Handbook of Experimental Pharmacology* (Erdös, E. G., ed.), pp. 250–288, Springer-Verlag, Berlin
Habermann, E., Klett, W. & Rosenbusch, G. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **332**, 121–142
Hamberg, U. (1959) *Biochim. Biophys. Acta* **34**, 135–146
Hamberg, U. (1969) *Scand. J. Clin. Lab. Invest.* **24**, 37–47
Hamberg, U. & Tallberg, Th. (1972) *J. Immunol. Methods* **2**, 17–24
Hamberg, U., Elg, P., Nissinen, E. & Stelwagen, P. (1975) *Int. J. Protein Res.* in the press
Kato, T., Suzuki, T., Ikeda, K. & Hamaguchi, K. (1967) *J. Biochem. (Tokyo)* **62**, 591–598
Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254
Nagasawa, S., Mizushima, Y., Sato, T., Iwanaga, S. & Suzuki, T. (1966) *J. Biochem. (Tokyo)* **60**, 643–652
Ouchterlony, O. (1948) *Ark. Kemi Mineral. Geol.* **26B**, no. 14, 1–9
Pierce, J. V. (1968) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **27**, 52–57
Pierce, J. V. (1970) in *Handbook of Experimental Pharmacology* (Erdös, E. G., ed.), pp. 21–51, Springer-Verlag, Berlin
Pierce, J. V. & Webster, M. (1966) in *Hypotensive Peptides* (Erdös, E. G., Back, N. & Sicuteri, F., eds.), pp. 130–138, Springer-Verlag, New York
Schultze, H. E. & Heremans, J. F. (1966) *Molecular Biology of Human Proteins*, vol. 1, p. 207, Elsevier Publishing Co., Amsterdam
Spiro, R. G. (1966) *Methods Enzymol.* **8**, 15–16
Spragg, J. & Austen, F. (1971) *J. Immunol.* **107**, 1512–1519
Spragg, J., Kaplan, A. P. & Austen, F. (1973) *Ann. N.Y. Acad. Sci.* **209**, 372–386
Wuepper, K. D. & Cochrane, C. G. (1971) in *Biochemistry of the Acute Allergic Reactions* (Austen, F. & Becker, E. L., eds.), pp. 299–320, Blackwell Scientific Publications, Oxford