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Purification and Characterization of Two Human Erythrocyte Arylamidases Preferentially Hydrolysing *N*-Terminal Arginine or Lysine Residues

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Two arylamidases (I and II) were purified from human erythrocytes by a procedure that comprised removal of haemoglobin from disrupted cells with CM-Sephadex C-50, followed by treatment of the haemoglobin-free preparation subsequently with DEAE-cellulose, gel-permeation chromatography on Sephadex G-200, gradient solubilization on Celite, isoelectric focusing in a pH gradient from 4 to 6, gel-permeation chromatography on Sephadex G-100 (superfine), and finally affinity chromatography on Sepharose 4B covalently coupled to L-arginine. In preparative-scale purifications, enzymes I and II were separated at the second gel-permeation chromatography. Enzyme II was obtained as a homogeneous protein, as shown by several criteria. Enzyme I hydrolysed, with decreasing rates, the L-amino acid 2-naphthylamides of lysine, arginine, alanine, methionine, phenylalanine and leucine, and the reactions were slightly inhibited by 0.2 M-NaCl. Enzyme II hydrolysed most rapidly the corresponding derivatives of arginine, leucine, valine, methionine, proline and alanine, in that order, and the hydrolyses were strongly dependent on Cl^-. The hydrolysis of these substrates proceeded rapidly at physiological Cl^- concentration (0.15 M). The molecular weights (by gel filtration) of enzymes I and II were 85000 and 52500 respectively. The pH optimum was approx. 7.2 for both enzymes. The isoelectric point of enzyme II was approx. 4.8. Enzyme I was activated by Co^{2+}, which did not affect enzyme II to any noticeable extent. The kinetics of reactions catalysed by enzyme I were characterized by strong substrate inhibition, but enzyme II was not inhibited by high substrate concentrations. The Cl^--activated enzyme II also showed endopeptidase activity in hydrolysing bradykinin.

Human erythrocytes contain several peptidohydrolases, for which the following biological roles can be suggested. (1) They effect the degradation of erythrocyte proteins and peptides at the end of the cell's life span. (2) They contribute to the liberation of active substances from the erythrocytes in serum under certain pathological conditions. (3) They affect the metabolism of peptides (reduced glutathione, for example) involved in the maintenance of the rigidity of the cell wall. Preliminary evidence (Mäkinen, 1975) suggests that the human erythrocyte arylamidases include an enzyme that in some aspects resembles the Cl^--activated aminopeptidase B (arginine aminopeptidase, EC 3.4.11.6) described in the rat and many other animal tissues (Hopsu *et al.*, 1966*a*,*b*; Mäkinen, 1975).

The present report describes a purification method for two arylamidases found in human erythrocytes. Both enzymes hydrolyse L-amino acid 2-naphthylamides of various amino acids, the best substrate being the 2-naphthylamides of either arginine or lysine. These enzymes (I and II) differ from each other with regard to a property that may be decisively important in their function *in vivo*. Enzyme I is slightly inhibited by NaCl at physiological concentrations, whereas the activity of enzyme II is almost absolutely dependent on the presence of Cl^- or certain other anions. It has been suggested that activation of a proteolytic enzyme by 0.9% NaCl indicates extracellular function of the enzyme (Mäkinen, 1975). The present results should also be considered in the light of the fact that the concentration of Cl^- in erythrocytes is considerably higher than in most other tissue cells or intracellular compartments.

Materials and Methods

Chemicals

The L-amino acid 2-naphthylamides, L-prolyl-L-phenylalanine amide hydrochloride, L-prolyl-L-lysine hydrate, poly(L-lysine), poly(L-hydroxyproline), poly(L-proline), L-leucyl-L-leucine, hippuryl-L-lysine, hippuryl-L-arginine, bovine haemoglobin and dithiothreitol were purchased from Schwarz–Mann, Orangeburg, NY, U.S.A. 3,3-Dimethylglutaric acid,

L-arginine, DL-alanine, DL-serine, L-leucine and L-proline were obtained from Fluka, Buchs SG, Switzerland. Bradykinin was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Bovine serum albumin, glycylglycine amide acetate, L-leucylglycine, glycyl-DL-alanine, glycyl-L-isoleucine, ε-amino-n-hexanoic acid, 1-naphthyl acetate, 1-naphthyl propionate, 1-naphthyl butyrate and 2-naphthylamine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Fast Garnet GBC Salt and Coomassie Blue were from G. T. Gurr, London, U.K. The following compounds were obtained from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.: DL-leucine, DL-hydroxyproline, DL-tyrosine, DL-phenylalanine, L-methionine and L-lysine hydrochloride. Sodium *p*-chloromercuribenzoate was purchased from Calbiochem, Los Angeles, CA, U.S.A., and L-ornithine from British Drug Houses, Poole, Dorset, U.K. Sephadex G-25, G-100 (superfine), Sepharose 4B, CM-Sephadex C-50 and Blue Dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholine ampholytes were obtained from LKB-Produkter, Bromma, Sweden. DEAE-cellulose was from Schleicher und Schüll, Dassel/Kr., Einback, Germany. Celite 545 (silicate content 89%) was obtained from Johns–Manville, Lompoc, CA, U.S.A. All other chemicals were from E. Merck A.-G., Darmstadt, Germany.

Buffers

Phosphate buffers were made with KH_2PO_4 by adjusting the pH with 2M-NaOH. Boric acid/borax buffers were prepared from $Na_2B_4O_7,10H_2O$ by adjusting the pH with 0.2M-H_3BO_3. 3,3-Dimethylglutarate buffers were made from 3,3-dimethylglutaric acid and 2M-NaOH. 1.0M-Acetate buffer was made of 1.0M-sodium acetate and acetic acid.

Column chromatography

All column-chromatographic procedures were carried out at 4°C. Gel-permeation chromatography was carried out according to the instructions of the supplier of the gels. Sepharose 4B covalently coupled to amino acids (L-arginine, L-lysine, L-ornithine, L-proline, DL-phenylalanine, L-methionine or L-leucine), used in affinity chromatography, was prepared as described by Cuatrecasas & Anfinsen (1971). Isoelectric focusing was carried out with an LKB 8101 electrofocusing column (110ml), equipped with the LKB gradient mixer. The instructions given in the instruction manual of the supplier of the device were followed, with the exception that the upper-electrode solution was omitted. The column was water-jacketed at 4°C. The column was emptied by means of a peristaltic pump at a flow rate of 0.2–0.3ml/min through the Uvicord II Ultraviolet Absorptiometer reading at 278nm. The pH of the fractions was immediately measured at 4°C with a Beckman Combination Electrode 39030.

Celite chromatography with decreasing $(NH_4)_2SO_4$ concentration gradient [$(NH_4)_2SO_4$ gradient solubilization] was carried out as described by King (1972). Concentration of protein solutions was carried out with the Amicon ultrafiltration system TCF-10 (Amicon B.V., Oesterhout N.B., Holland) with UM 20E membranes.

Analytical electrophoresis on polyacrylamide gels was carried out at pH8.9 as described by Davis (1964) with 7.5% (w/v) acrylamide. The runs were carried out at a current of 5mA/tube for 3h. Each sample was applied on two separate gels (20μl/gel). One gel was stained for protein with 0.25% Coomassie Blue in methanol/acetic acid/water (4:1:4, by vol.). Excess of stain was removed electrophoretically by applying 10–15mA/tube in 5% (v/v) acetic acid solution. Enzyme activity was localized in the other gel as described earlier (Mäkinen & Mäkinen, 1972).

Erythrocytes

Human erythrocytes, collected from the blood of healthy subjects, were obtained in sterile, non-pyrogenic plastic bags (JA-5N Blood-Pack units, Fenval Division, Tranevol Laboratories, Brussels, Belgium) from the local Red Cross Blood Service. After the collection the cells were stored for 10–14 days at 4°C as 250g batches containing an anticoagulant solution. Each 100ml of cells contained 327mg of citric acid monohydrate, 2.63g of sodium citrate dihydrate, 222mg of Na_2HPO_4,H_2O and 2.32g of anhydrous D-glucose. After this storage the cells were harvested and washed by centrifugation as described below. The washing solution of the erythrocytes contained 0.9% NaCl, 0.1% glucose and 0.1mM-2-mercaptoethanol. Altogether 20kg of erythrocytes (fresh weight) was used in this study.

Enzyme assay

Arylamidase activity was assayed at 30°C in reaction mixtures (0.6ml) containing the following ingredients: 0.3ml of buffer, 0.1ml of substrate solution, 0.1ml of water or aqueous solution of a compound whose effect on the reaction was tested, and finally 0.1ml of enzyme solution. The substrate solutions were prepared from various L-amino acid 2-naphthylamides by dissolving the solid substance in 5ml of ethanol in a 100ml volumetric flask and making up to 100ml with water. The final substrate concentrations used were constantly 0.167mM, except for kinetic experiments for which the concentrations are given separately. The working substrate was L-arginine 2-naphthylamide. The velocity of the hydrolysis of the substrates at 30°C was measured by arresting the reaction after a constant time (usually 60min) by adding 0.2ml of 1.0M-acetate buffer, pH4.2, which contained 0.1%

diazotized 4-amino-1,3'-dimethylazobenzene (Fast Garnet GBC Salt) and 10% (v/v) Tween-20. The absorbance of the resulting azo-dye was measured at 525nm. The hydrolysis of 1-naphthyl esters was determined in reaction mixtures described above, but the reactions were terminated by first adding the diazonium compound (in water), and then by acidifying with the acetic acid/Tween mixture 10min later. The absorbance, which depended on the 1-naphthyl ester used, was determined at 500–550nm.

Effect of chemical compounds

The effect of various chemical compounds (metal chlorides, KF, NaSCN, EDTA, sodium *p*-chloromercuribenzoate, L-arginine and L-lysine) on enzyme activity was tested in reaction mixtures as described above. For metal chlorides, the enzymes were first incubated for 15min at 30°C in mixtures containing buffer and effector. The substrate was then added and the reactions were carried out for 60min at the same temperature. With other compounds no preincubation was carried out. The enzymes were dialysed for these experiments at 4°C for 24h against water.

Hydrolysis of bradykinin and other peptides

The ability of enzyme II to hydrolyse bradykinin was normally studied in reaction mixtures (60μl) consisting of 20μl of a bradykinin solution (usually 2mg/0.2ml of water), 20μl of 0.01M-phosphate buffer, pH7.2, and 20μl of enzyme II. Appropriate control mixtures, omitting either enzyme or bradykinin, were prepared. The mixtures were incubated for various times (2–20h) at 30°C in small (1ml) tubes sealed with Parafilm. The mixtures were then chilled quickly in an iced-water bath and 20–40μl samples of the reaction mixtures were applied on 0.5mm-thick silica-gel layers made from gel slurry containing 0.2M-sodium acetate (30g/10ml; Randerath, 1964). The thin-layer plates were developed in butanol/acetic acid/water (3:1:1, by vol.) and stained with ninhydrin. The stained spots were analysed with a Chromoscan integrating and recording densitometer (Joyce, Loebl and Co., Gateshead, Tyne and Wear, U.K.). Reaction mixtures 5 times more concentrated with regard to each of the above constituent solutions were also analysed with the Beckman Unichrome amino acid analyser by the standard procedure (Beckman Manual, 1965). When the effect of chemical compounds on the hydrolysis of bradykinin was studied, 20μl of an aqueous solution of the compound involved was added to the reaction mixture (80μl final volume).

The hydrolysis of other peptides was studied in reaction mixtures similar to those mentioned above. With di- and tetra-peptides the final concentrations of the substrates added ranged from 0.167 to 1.0mM and the reaction times from 1 to 17h. One-dimensional t.l.c. was carried out as described above, but in addition to the development in acidic butanol, phenol/water (3:1, w/w) was also used. With polyamino acids, amounts of the substrate (0.2–1.0mg) were suspended directly in the reaction mixtures (60μl). The hydrolysis of hippuryl amino acids was carried out by the ninhydrin method of Yemm & Cocking (1955). The hydrolysis of denatured haemoglobin was carried out as suggested for the determination of trypsin (Anson & Mirsky, 1933; Anson, 1938).

Determination of NH_4^+ and protein

Ammonium ions in chromatographic fractions were assayed by the method of Searsy *et al.* (1961). The method of Lowry *et al.* (1951) was normally used to determine the protein concentrations with bovine serum albumin as standard. In certain fractionations the protein concentrations were determined at 280nm and in isoelectric focusing the Uvicord Absorptiometer measured the protein at 278nm. However, when the concentrations of the proteins were very low, as in the last fractionation step of the purification method (affinity chromatography), the proteins were determined by measuring the absorbance of the fractions at 220nm, with serum albumin as standard.

Molecular-weight determination by gel filtration

A column (140cm×1.6cm) of Sephadex G-100 (superfine) was equilibrated with the elution buffer, 10mM-3,3-dimethylglutarate, pH7.2. The sample contained (in 2.0ml) the studied enzymes (I or II), 0.2ml of 0.1% Blue Dextran solution and 2mg each of the standard proteins (mol.wt.): γ-globulin (160000), serum albumin (67000), ovalbumin (45000), chymotrypsinogen (25000) and myoglobin (17800). Fractions of volume 1.0ml were collected. The standard proteins were determined by the method of Lowry *et al.* (1951); myoglobin was also measured at 405nm. The standard proteins (excluding γ-globulin) gave a straight line when elution volume was plotted against the logarithm of molecular weight.

Amino acid analyses

The amino acid composition of the Cl^--dependent enzyme II was determined by the standard procedure described in the Beckman Manual (1965) for the Beckman Unichrom amino acid analyser. The enzyme preparations were treated for the analyses as follows. Enzyme (15ml), obtained from the final step of the purification procedure (affinity chromatography), was dialysed for 24h at 4°C against 10litres of 0.1mM-3,3-dimethylglutarate buffer, pH7.2, containing 1.0mM-2-mercaptoethanol.

The dialysis solution was changed after 12h. Three separate 15ml portions of the enzyme were

dialysed in the above way. During the dialysis the 15ml volume was increased by 2.5–3ml. The three portions were pooled (55ml) and analysed for protein (at 220nm), enzyme activity and amino acids. For the amino acid analyses the 55ml of the dialysed enzyme solution was first concentrated by freeze-drying to 2ml, mixed with 2ml of 11.6M-HCl and heated for 24h at 110°C in sealed glass ampoules. After this step, the procedure was exactly as described in the Beckman Manual. The whole procedure was repeated twice with other sets of 15ml portions of the enzyme resulting from separate purifications. The dialysed material contained 40–43 μg of protein/ml. The dialysis lowered the enzyme activity each time by approx. 65%.

The amount of tryptophan was evaluated from the tyrosine/tryptophan molar ratio by the method of Bencze & Schmid (1957), which was based on the determination of the u.v. absorption spectrum of purified enzyme II. The value used for tyrosine was obtained from the amino acid analyses described above. The spectrum was determined with a Hitachi Perkin–Elmer model 124 spectrophotometer at 25°C with the step-9 purified enzyme. The enzyme for the determination of the spectrum was obtained from the last purification step.

Results

Purification procedure

Preliminary experiments showed that the erythrocytes of various animal species contain several arylamidases, one of which, to a certain extent, resembled the true aminopeptidase B of rat liver. A method for the purification of this and one additional human erythrocyte arylamidase is described below. During the purification enzyme activity was measured in 0.025M-phosphate buffer. All steps of the purification procedure were carried out at 4°C.

1. *Washing and disruption of erythrocytes.* The contents of the erythrocyte bags were centrifuged for 5min at 43500$g_{av.}$. The supernatant fluid and the whitish cell layer above the erythrocytes were withdrawn with a suction pump.

Batches of approx. 250g of erythrocytes (wet wt.) were then washed with 1000ml of washing solution to remove leucocytes and other unwanted material. The mixture was stirred for 5min with a glass rod and centrifuged for 5min at 43500$g_{av.}$. The leucocytes and other material that remained above the erythrocyte mass were removed by suction. The washing was repeated twice. Approx. 350–400ml of washed erythrocyte mass was obtained from the treatment of each 250g cell batch. The cells were then disrupted by adding cold 0.1mM-2-mercaptoethanol to a final 4-fold volume (250ml of cell mass and 750ml of water). The mixture was stirred for 15min and then centrifuged for 20min at 43500$g_{av.}$. The clear red supernatant fluids were carefully decanted to avoid loosening of the pellets. The pH of the combined supernatant fluids was adjusted to 7.5 with 1M-NaOH. Altogether 80litres of haemolysate was prepared for the purification procedure.

2. *Removal of haemoglobin.* The 80litres of haemolysate resulting from the previous step was added in 1litre portions to a slurry of CM-Sephadex C-50, equilibrated with 0.01M-3,3-dimethylglutarate buffer, pH6.5, which contained 0.1mM-2-mercaptoethanol. The ratio of haemolysate to slurry was 1:2.5. The mixture was stirred with a glass rod and allowed to stand for 30min, after which the thick, red-coloured gel was transferred to a Whatman filter-paper disc (no. 1) in a 4-litre Buchner funnel. The transfer was accomplished with the same buffer. The filtrate was removed from the gel with suction and collected in a 250ml glass cylinder placed on the bottom of the suction flask. The resulting enzyme solutions were light brown and contained approx. one-eightieth of the original amount of protein. The amount of gel required to bind haemoglobin and to yield a light-brown or almost colourless solution could be easily controlled by observing the colour of the filtrate. If some red colour was transferred through the gel into the cylinder, additional CM-Sephadex gel was used to decolorize the enzyme solution. The volume of the enzyme preparation was either increased by approx. 10% in the CM-Sephadex treatment or it remained practically unchanged. The 80litres of haemolysate thus resulted in 85litres of light-brown and essentially haemoglobin-free solution. Enzymes I and II were not bound to CM-Sephadex C-50 under the conditions described.

3. *Concentration.* The 85 litres of solution resulting from the previous step were concentrated in approx. 10 litre portions with the Amicon ultrafiltration system. Each step decreased the volume to 140–145ml. A final enzyme solution of 1200ml was thus obtained from an initial volume of 85 litres. This 1200ml was divided into four portions, each of 300ml, which were treated separately in the next step.

4. *Treatment of DEAE-cellulose involving stepwise de-adsorption with NaCl.* Portions (300ml) of the enzyme solution resulting from the previous step were mixed in a beaker with 450ml of DEAE-cellulose slurry. A fraction of dry DEAE-cellulose was sieved (100–200 mesh) for this purpose. A slurry was made with an equal volume of 0.05M-phosphate buffer, pH6.7, which contained 0.1mM-2-mercaptoethanol. Before the addition of the enzyme preparation to the cellulose, excess buffer was removed. The mixture was transferred after standing for 30min to a 2-litre Buchner funnel (Whatman filter paper no. 1) and non-adsorbed proteins were removed by suction (filtrate no. 1). Then 1500ml of 0.07M-NaCl solution was stirred into the cellulose,

Table 1. *Adsorption of enzymes I and II to DEAE-cellulose*

The adsorption of enzymes I and II to DEAE-cellulose was carried out in 1 mM-phosphate buffer, pH 6.7 (the enzymes did not separate at this stage). The Table gives the specific activities (L-arginine 2-naphthylamide as substrate) of the enzyme preparations (approx. 1000ml filtrates), determined in the presence of 0.2M-NaCl. All values represent medians obtained from ten separate operations.

Step	Designation of enzyme preparation	Specific activity (nmol/min per mg of protein)	Protein (mg/ml)
Pooled concentrates from step 3		17	30
After mixing with cellulose	1	0*	7
After addition of 0.07M-NaCl	2	0.6	1.5
After addition of 0.25M-NaCl (final concn. 0.322M)	3†	25	1.8
After addition of 0.25M-NaCl (final concn. 0.572M)	4†	26	0.5
After addition of 1.0M-NaCl (final concn. 1.572M)	5†	10	0.15

* The enzymes were adsorbed to the cellulose resulting in practically nil activity in the filtrate.

† Preparations 3 and 4 were regularly chosen for step 5 of the purification procedure. Owing to their low protein content, preparations designated as 5 were also used when their specific activity exceeded 1nmol/min per mg of protein.

the outlet of the funnel being kept closed. After being left for 30min, filtrate no. 2 was removed by suction. This contained only small quantities of enzymes I and II. Next, 1500ml of 0.25M-NaCl was mixed with the cellulose and left to stand for 30min with constant agitation. Filtrate (no. 3) was removed by suction. Another 1500ml volume of 0.25M-NaCl was then mixed with the cellulose and filtrate no. 4 was obtained by suction. The third addition of 1500ml of 1.0M-NaCl produced filtrate no. 5. An equilibrium period of 30min with thorough mixing with a glass rod followed each addition. Table 1 gives the specific activities and protein concentrations of the enzyme preparations collected. Solutions designated as 3 and 4 (comprising altogether approx. 3litres) were used in step 5. Both enzymes I and II behaved similarly in the conditions described and they were thus recovered in the final filtrate. The adsorption and de-adsorption of both enzymes were monitored in small samples of the filtrates. In all cases the bulk of the enzymes appeared in the 0.07–0.57M-NaCl fraction. Supernatant no. 5 always contained only small amounts of the enzymes studied. The 3-litre solutions were separately concentrated by the Amicon ultrafiltration system to 350ml. The next step was commenced after the treatment of the whole enzyme solution of step 3 with DEAE-cellulose. Four separate batch operations were necessary (4×350ml=1400ml).

5. *Gel-permeation chromatography I.* Separate portions (80–110ml) of the enzyme solution resulting from the previous step were fractionated through a Sephadex G-200 column (8.7cm×100–105cm; gel volume approx. 6-litres). Thirteen separate operations were necessary to treat the material obtained from step 4. Both enzymes were eluted together in a volume from 1900 to 2800ml (Fig. 1). The most active fractions were pooled and the resulting solution (800ml) was concentrated to 55–60ml. The concentrates were pooled (750ml). When this step was carried out on an analytical scale (column dimensions 1.5cm×140cm) with small samples (1–2ml), enzymes I and II were separated.

6. *Gradient solubilization on Celite.* The concen-

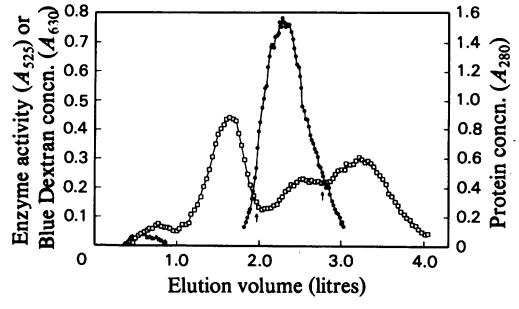


Fig. 1. *Preparative gel-permeation chromatography (descending) of erythrocyte arylamidases on Sephadex G-200 gel (step 5)*

The column dimensions were 105.5cm×8.7cm (6.27litre) and elution buffer was 0.01M-3,3-dimethylglutarate buffer, pH 7.2, containing 1.0mM-2-mercaptoethanol, with a hydrostatic pressure of 18cm and flow rate of 1.2ml/min (temperature, 4°C). The fraction volume was 18ml. A sample (110ml) of concentrated enzyme from the DEAE-cellulose step was chromatographed (specific activity 20 nmol/min per mg of protein in 0.2M-NaCl and 12nmol/min per mg of protein without added salt). Blue Dextran solution (0.1%, 3ml) was mixed into the enzyme preparation before application. Symbols: ●, enzyme activity tested in the presence of 0.2M-NaCl; □, protein; △, Blue Dextran (A_{630}). The fractions between the arrows were regularly combined for concentration and subjected to step 6.

trates resulting from the previous step were mixed with Celite 545, suspended in 0.01 M-3,3-dimethylglutarate buffer/5 mM-2-mercaptoethanol, pH 7.2. The suspensions had the following composition: 250 ml of enzyme solution (containing 3.2 g of protein, from step 5), 100 g of Celite 545 and buffer to a final volume of 2000 ml. The ingredients were thoroughly mixed with a magnetic stirrer and 1080 g of $(NH_4)_2SO_4$ was added. The mixture was stirred for 60 min and packed into a chromatographic column (3.0 cm × 70 cm; Fig. 2) with a peristaltic pump connected to the bottom outlet of the column. The suspension, placed in a funnel on the top of the column, was constantly agitated with a propeller. After packing, a linearly decreasing $(NH_4)_2SO_4$ gradient from 80 to 40% was applied on the column. The gradient solutions were of equal weight (1230 g). The shape of the $(NH_4)_2SO_4$ gradient was determined with a Metrohm Konduktometer E 382 (Metrohm, Herisau, Switzerland), portions (100 μl) of the fractions being diluted 100-fold with water. An effective purification of the two erythrocyte enzymes was achieved. Three identical large-scale Celite fractionations were carried out on the enzyme preparation resulting from step 5 (3 × 250 ml = 750 ml). The fractionations resulted in 1100 ml of combined enzyme solution, which contained both enzyme I and enzyme II. This solution was concentrated by ultrafiltration to 550 ml. The high amount of $(NH_4)_2SO_4$ prevented more effective concentration. The rest of the salt was then removed from the 550 ml concentrate on Sephadex G-50 columns (8.7 cm × 40 cm) eluted with 0.01 M-3,3-dimethylglutarate buffer, pH 7.2. The 550 ml volume was treated with the Sephadex G-50 column in five parts. The complete removal of $(NH_4)_2SO_4$ from the pooled fractions was completed by dialysis (against a 5–6-fold volume of the above buffer). The dialysed solution was finally concentrated by Amicon to 360 ml. These procedures did not decrease the enzyme activity.

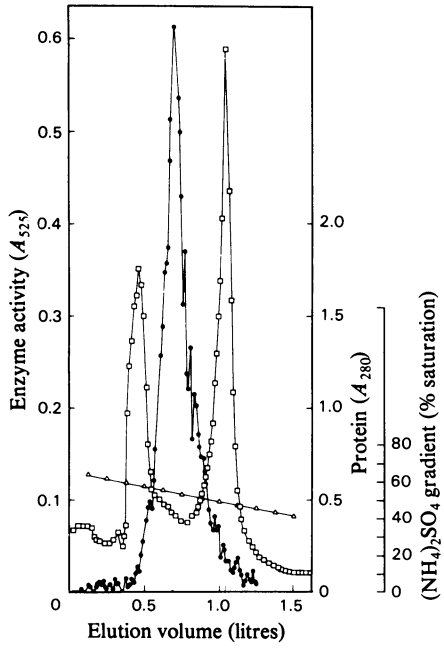


Fig. 2. *Preparative separation of erythrocyte arylamidases by $(NH_4)_2SO_4$-gradient solubilization (step 6)*

The column dimensions were 3.0 cm × 70 cm (0.495 litre) and the column was packed with a mixture of Celite, enzyme preparation and $(NH_4)_2SO_4$ in 0.01 M-3,3-dimethylglutarate buffer, pH 7.2, which contained 5 mM-2-mercaptoethanol. The elution gradient was linear with respect to $(NH_4)_2SO_4$ and was from 80% to 40% saturation in 5 mM-2-mercaptoethanol. The hydrostatic pressure was 30 cm, with a flow rate of 1.0 ml/min and temperature of 4°C. The fraction volume was 12.5 ml. Sample (250 ml of concentrated enzyme solution from step 5, treated as described in the text) was loaded on the column. Symbols: ●, enzyme activity tested in the presence of 0.2 M-NaCl; □, protein; △, $(NH_4)_2SO_4$ concentration. The fractions between 56% and 51% were regularly combined for removal of salt, concentrated and subjected to step 7.

7. *Isoelectric focusing.* The desalted enzyme solution (360 ml) was subjected in four separate portions to preparative isoelectric focusing in a pH gradient from 4 to 6. A representative fractionation is shown in Fig. 3. The active fractions from each run were pooled, the pH of the solution was adjusted to 7.0 with 0.1 M-NaOH, the ampholytes were removed and the volume of the combined focusing pool was decreased by ultrafiltration to 140 ml. Up to this preparative stage the two arylamidases overlapped in all fractionations, but their partial separation can be observed in Fig. 3. When used on an analytical scale (with 3 ml sample) this step separated the enzymes.

8. *Gel-permeation chromatography II.* Preliminary experiments carried out on a small scale with crude haemolysates showed that chromatography carried out with sufficiently small volumes and protein concentrations on a column of Sephadex G-100 (superfine) resulted in good separation of the two enzymes. This information was exploited at this stage of purification by using a column with inner dimensions of 4.8 cm × 118 cm (2.1 litres). A representative chromatogram is shown in Fig. 4. Two arylamidases were separated. The one with higher molecular weight (enzyme I) was slightly inhibited by 0.2 M-NaCl, whereas the activity of the other enzyme (enzyme II) was clearly dependent on NaCl, when L-arginine 2-naphthylamide was used as substrate. The active fractions were pooled and both enzyme solutions were concentrated. The fractionation was repeated to obtain enough material for the last purification

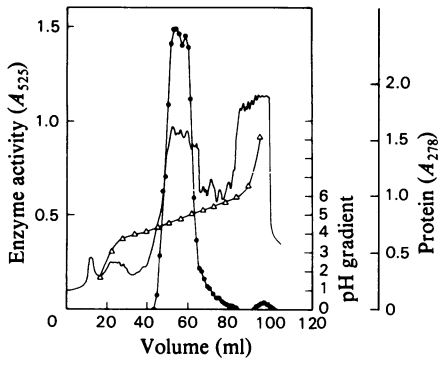


Fig. 3. *Preparative isoelectric focusing of erythrocyte arylamidases (step 7)*

The column volume was 110ml and the pH gradient was from 4 to 6 with 1% ampholyte concentration. A current of 10mA (500V) was applied at the start of the focusing and 1mA (530V) at the end. The cathode was on the top of the column (the upper electrode solution was omitted). Focusing time was 47h with a temperature in the jacket of 4°C and fraction volume of 1.4ml. A 90ml sample of enzyme resulting from step 6 was used. Symbols: ——, protein; ●, enzyme activity tested in the presence of 0.2M-NaCl; △, pH gradient. Fractions between 45 and 65ml were regularly pooled and subjected to step 8.

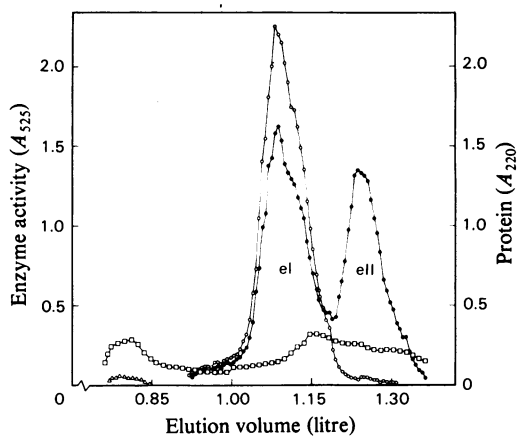


Fig. 4. *Preparative molecular permeation chromatography (descending) of erythrocyte arylamidases on Sephadex G-100 (superfine) (step 8)*

The column dimensions were 4.8cm×118cm (2.13 litres) with an elution buffer of 0.01M-3,3-dimethylglutarate, pH7.2, containing 1mM-2-mercaptoethanol. The hydrostatic pressure was 22cm (temperature 4°C). A 36.5ml sample of enzyme from step 7, mixed with 1.6ml of 0.1% Blue Dextran was applied to the column at a flow rate of 1.0ml/min and fractions of volume 3.0ml were collected. Symbols: ●, enzyme activity with 0.2M-NaCl; ○, enzyme activity without added salt; □, protein; △, Blue Dextran (A_{630}). The fractions between 1.03 and 1.16litre (enzyme I) and between 1.21 and 1.31litre (enzyme II) were regularly pooled. Pooled volumes of enzyme II were concentrated for the final step.

step. The enzyme designated as I was, however, considered pure enough for most enzyme studies. Because enzyme II resembled, in its Cl^- effects, the true aminopeptidase B, this enzyme was investigated more thoroughly. Enzyme II was thus finally purified in step 9.

9. *Affinity chromatography.* Enzyme II was obtained as a homogeneous protein by affinity chromatography on Sepharose 4B covalently coupled to L-arginine. The optimum conditions are shown in Fig. 5. No concentration or precipitation was necessary to keep the enzyme active, even during prolonged storage in the rather diluted NaCl-containing solution resulting from affinity chromatography.

Final treatment of enzymes. Enzyme I was concentrated approx. 10-fold by ultrafiltration after preparative molecular-permeation chromatography on Sephadex G-100 (superfine) (Fig. 4). The enzyme solution thus contained 0.01M-3,3-dimethylglutarate buffer and 1.0mM-2-mercaptoethanol. Enzyme II was not concentrated after affinity chromatography. The enzyme preparation contained 0.01M-3,3-dimethylglutarate buffer, 1.0mM-2-mercaptoethanol and approx. 0.5M-NaCl. Both enzymes were stored in these states at 4°C for several years. For the assays of activity enzyme I was diluted 250-fold and enzyme II was diluted 10-fold.

Specific activity. Table 2 shows the development of specific activity during purification. The results require the following considerations. (*a*) The calculation of the specific activity for the crude preparations was based on the fact that both enzymes I and II were simultaneously present in the reaction mixtures. Possibly other enzymes with low activity towards the substrate were also present. Hence the results given show the overall specific activity of the preparations toward L-arginine 2-naphthylamide until step 8, which separated the enzymes (Fig. 4). After this stage the specific activities of enzymes I and II are given separately. (*b*) The overall specific activity of the haemolysates in 0.2M-NaCl (step 1; Table 2) ranged in the 80 separate lots of erythrocytes from 0.32 to 0.52nmol/min per mg of protein. After the removal of haemoglobin the activity ranged from 21.3 to 42.0nmol/min per mg of protein, a 70–80-fold purification of the mixture of enzymes I and II. Certain later steps (ultrafiltration and storage before step 6), however, destroyed the enzymes to a considerable extent, greatly affecting the yield.

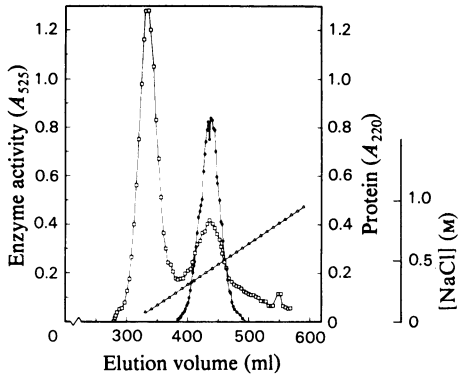


Fig. 5. *Preparative affinity chromatography (descending) of enzyme II (the Cl^--dependent enzyme; step 9)*
The column (2.1cm×96cm; 0.332 litre) was Sepharose 4B–arginine and the elution buffer was 0.01M-3,3-dimethylglutarate, pH7.2, containing 1.0mM-2-mercaptoethanol containing a NaCl gradient from 0 to 2.0M (mixing volume 300ml plus 300ml). The hydrostatic pressure was 20cm (temperature 4°C) and fractions of volume 2.0ml were collected at a flow rate of 0.5ml/min. A 2.0ml sample of the concentrated enzyme from step 8 was applied. Fractions from 410 to 480ml were regularly pooled. Symbols: ●, enzyme activity tested in the presence of 0.2M-NaCl; □, protein; △, NaCl.

Criteria of purity of enzyme II

The purity of enzyme II was established by analytical discontinuous-polyacrylamide-gel electrophoresis and by rechromatography on Sephadex G-100 (superfine), DEAE-cellulose and arginine–Sepharose 4B. The purity of 13 separate enzyme preparations was studied by these methods. In each case the peaks corresponding to enzyme activity and protein were coincident. The peaks were, within the limits of errors in enzyme and protein assays, homogeneous. No tailing was observed. The purified enzyme also produced a single homogeneous enzyme and protein peak in isoelectric focusing in the following pH gradients: 5–7, 4–6 and 3.5–5.0. In discontinuous-gel electrophoresis the enzyme band moved 52.5% of the length of the gel in 3h at a current of 5mA/tube (at 4°C).

Fresh solutions of the purified enzyme II did not contain measurable amounts of other nitrogen-containing compounds. This was shown by fractionating 1ml of the enzyme through a Sephadex G-25 column (2cm×37cm) and by analysing the fractions for NH_4^+ and ninhydrin-positive compounds. Only the fractions containing the enzyme reacted positively. The behaviour of enzyme II in affinity chromatography on arginine–Sepharose 4B (Fig. 5) also indicated high purity of the enzyme. Amino acid analyses carried out on two separate lots of enzyme

Table 2. *Purification of enzymes I and II from human erythrocytes*
The enzyme activity of the preparations was determined with 0.166mM-L-arginine 2-naphthylamide in 0.05M-phosphate buffer, pH7.2, carried out in either the presence or absence of 0.2M-NaCl. I and II refer to enzymes I and II, which were separated at the eighth purification step. The enzyme activities and protein concentrations are median values representing several separate lots of enzyme preparations treated during the entire purification procedure.

Purification step	No. of separate lots treated	Total volume (ml)	Total protein (g)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)		v_{NaCl}/v_{H_2O}
					Without added salt	0.2M-NaCl	
1. Erythrocyte haemolysate	80	80000	5200	2288000	0.22	0.44	2.04
2. After removal of haemoglobin	80	85000	68	1700000	14.4	25.0	1.74
3. After concentration	8	1200	37.6	752000	14.8	20.0	1.42
4. After adsorption to DEAE-cellulose, de-adsorption and concentration	4	1400	25.5	688500	16.4	27.0	1.64
5. After Sephadex G-200 chromatography	13	750	12.5	600000	29.4	48.0	1.63
6. After gradient solubilization [after removal of $(NH_4)_2SO_4$]	3	360	2.0	190000	87.0	95.0	1.20
7. After isoelectric focusing (after concentration)	4	140	0.8	88000	91.0	110.0	1.20
8. After Sephadex G-100 chromatography (before concentration)	4 I	1500	0.2	50800	268	254	0.95
	II	1500	0.2	24000	0	120	
9. After affinity chromatography (not concentrated)	13 II	650	0.02	3800	0	190	

II yielded essentially similar amino acid compositions in both analyses. From these findings, enzyme II was considered to be homogeneous.

Enzyme characteristics

Isoelectric point. The isoelectric point of enzyme II was determined by analytical isoelectric focusing with Ampholine ampholytes in three pH gradients at 0°C. The isoelectric points and gradients were: 4.8 (5–7), 4.8 (3.5–5.0) and 4.7 (4–6). No distinct micro-heterogeneity of enzyme II was observed in any of the pH gradients when the enzyme activity was tested with the 2-naphthylamide derivatives of L-arginine and L-leucine. The focusings were carried out 3–4 weeks after the completion of the purification procedure. The isoelectric point of enzyme I was not determined.

Stability. Both enzyme I and enzyme II, after completion of the whole purification procedure, were very stable in aqueous solution at 4°C. Both enzymes could be stored for several months in this way without any significant loss of enzyme activity. Freezing lowered the activity of enzyme II even in the presence of thiol compounds and NaCl.

Storage of enzyme II for 1 year at 4°C after affinity chromatography did not result in the same type of age-dependent aggregation as observed with rat liver aminopeptidase B (Mäkinen, 1972). However, slight micro-heterogeneity was detected in molecular-exclusion chromatography on Sephadex G-100 Superfine. The same micro-heterogeneity was not found with the crude enzyme preparations. During storage for 1 year some inactive proteins of high molecular weight were separated. The enzyme activity was concomitantly lower. Portions of enzyme II have been stored at 4°C in this laboratory for 4 years without total loss of enzyme activity. This prolonged storage gave, on Sephadex G-100 (superfine) columns, one new Cl^--activated form of the enzyme. The new form had about one-quarter of the activity of the original form (as deduced from peak areas). The molecular weight of the new form was twice that of the freshly purified enzyme.

Molecular weight. The molecular weights of freshly purified enzymes I and II were determined by molecular-permeation chromatography on a column of Sephadex G-100 (superfine) by using commercial proteins as standards. The mean value of the molecular weight from four runs was 85000 ± 9200 (S.D.) for enzyme I and 52500 ± 4500 for enzyme II (the Cl^--dependent enzyme). No evidence was obtained about the existence of subunits of freshly prepared enzymes I or II, which would have been revealed by the chromatographic methods. It is obvious that both enzymes are composed of one single unit, although aged enzyme II was found to contain a form with higher molecular weight, brought about by rather unphysiological conditions (4 years storage at 4°C).

Amino acid composition. The results obtained from the amino acid analyses of homogeneous step-9 enzyme II subjected to acid hydrolysis for 24h are presented in Table 3. The values shown for methionine and isoleucine were corrected, respectively, for methionine sulphoxide and alloisoleucine, which were formed to some extent during the course of hydrolysis. The results are the average of two values obtained with separate enzyme lots.

Substrate specificity. Amino acid 2-naphthylamides. Table 4 shows the substrate specificity of the starting material, serum and enzymes I and II, studied with several amino acid 2-naphthylamides. The specificities of the enzymes were almost equally broad, but they partly comprised different substrates and the dependence of the activity of enzyme II on Cl^- greatly increased differences between the two arylamidases. The main differences were the inability of enzyme I to hydrolyse L-valine 2-naphthylamide and the low rate of hydrolysis of L-lysine 2-naphthylamide by enzyme II under the conditions involved. In general, enzyme II had a very low activity in the absence of NaCl. L-Arginine 2-naphthylamide was hydrolysed by both enzymes at a high rate, but the L-lysine derivative was the best substrate of enzyme I. The corresponding L-ornithine derivative was not hydrolysed by either enzyme.

The effects of 0.2M-NaCl on the rate of the hydrolysis of L-arginine 2-naphthylamide and L-alanine

Table 3. *Amino acid composition of the Cl^--dependent arylamidase (enzyme II) of human erythrocytes*

The details of the amino acid analysis are given in the Materials and Methods section. Abbreviation: n.d., not determined.

	Amino acid (g/100g of protein)
Lys	8.6
His	3.4
Arg	5.0
Asp	10.0
Thr	6.2
Ser	9.8
Glu	13.2
Pro	5.7
Gly	6.0
Ala	5.3
½CyS	n.d.
Val	5.3
Met	0.6
Ile	4.0
Leu	9.7
Tyr	3.2
Phe	4.0
Trp	0.2–0.3

Table 4. *Substrate specificity of the haemolysate, serum and enzymes I and II*
Tested in 0.025M-phosphate buffer, pH7.2, either in the presence of 0.2M-NaCl or without added salt. Substrate concentrations: 0.166mM. The arithmetic means for three determinations are indicated. Abbreviations: 2-NNap, 2-naphthylamide; n.d., not determined.

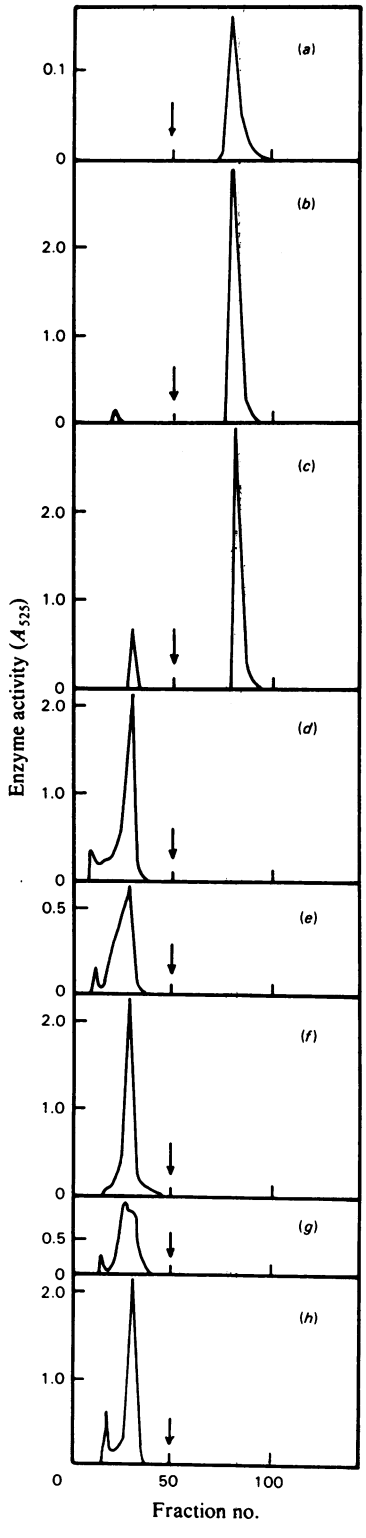
	Specific activity (nmol/min per mg of protein)							
	Haemolysate		Serum		Enzyme I		Enzyme II	
Substrate	No salt	0.2M-NaCl	No salt	0.2M-NaCl	No salt	0.2M-NaCl	No salt	0.2M-NaCl
L-Alanine 2-NNap	0.575	0.475	1.941	2.637	261.0	122.0	30.0	65.5
L-Arginine 2-NNap	0.435	0.730	0.542	1.197	267.8	254.5	0	190.0
α-Benzoyl-DL-arginine 2-NNap	0.025	0.030	0.061	0.028	0	0	0	0
Glycine 2-NNap	0.020	0.025	0.224	0.210	0	0	0	0
L-Histidine 2-NNap	0.005	0.005	0.056	0.042	0	0	0	
L-Hydroxyproline 2-NNap	0.002	0.002	0.035	0.023	0	0	0	0
L-Isoleucine 2-NNap	0.20	0.015	0.173	0.071	0	0	0	0
L-Leucine 2-NNap	0.185	0.225	0.748	1.314	121.5	120.8	28.5	142.5
L-Lysine 2-NNap	0.485	0.620	0.243	0.472	450.0	250.2	0	5.2
L-Methionine 2-NNap	0.360	0.350	1.110	1.777	260.5	200.5	9.6	118.0
L-Ornithine 2-NNap	n.d.	n.d.	n.d.	n.d.	0	0	0	0
L-Phenylalanine 2-NNap	0.535	0.440	0.154	0.093	250.2	80.5	0	93.0
L-Proline 2-NNap	0.025	0.025	0.077	0.077	3.1	2.5	0	112.1
L-Serine 2-NNap	0.005	0.005	0.107	0.042	0	0	0	2.8
L-Threonine 2-NNap	0.005	0.005	0.072	0.051	1.0	0	0	2.1
α-Tosyl-DL-arginine 2-NNap	0.025	0.005	0.010	0.010	0	0	0	0
L-Tryptophan 2-NNap	0.045	0.050	0.112	0.075	0	0	0	0
L-Tyrosine 2-NNap	0.080	0.085	0.072	0.047	0	0	0	0
L-Valine 2-NNap	0.005	0.005	0.075	0.065	0	0	11.5	135.0

2-naphthylamide catalysed by serum were opposite: the hydrolysis of the former substrate was strongly activated and that of the latter was inhibited. This selective NaCl effect has been considered an important characteristic of the biological fluids, other than serum, studied in these laboratories (Mäkinen, 1975). It is noticeable that the haemolysate had a low specific activity toward L-proline and L-valine 2-naphthylamides, although both were hydrolysed by enzyme II at a considerable rate.

Esters and hippuryl amino acids. Enzyme II did not cause detectable hydrolysis of 1-naphthyl acetate, 1-naphthyl propionate or 1-naphthyl butyrate when tested in 0.05M-phosphate buffer, pH6.2 and 7.2, in the presence of 0.2M-NaCl. The reaction time was 60min. Enzyme I hydrolysed the 1-naphthyl esters of acetic acid and propionic acid at a low rate under the above conditions (specific activity was less than 20nmol/min per mg of protein), but 1-naphthyl butyrate was hydrolysed at a still lower rate. NaCl (0.2M) slightly (by 10%) inhibited these hydrolysis reactions. Hippuryl-L-arginine or hippuryl-L-lysine was hydrolysed by enzyme II under the above conditions, but the rates were only one-tenth of those obtained with the corresponding 2-naphthylamide derivatives.

Peptides. The hydrolysis of bradykinin and a number of other peptides by enzyme II was studied. Bradykinin was found to be a substrate of this enzyme. It was not possible at this stage of the study to characterize the hydrolysis products, but the cleavage of different peptide bonds of bradykinin most likely occurred stepwise as a function of time: particular peptide bonds (most likely Pro–Pro and Gly–Phe) were hydrolysed at the first stage. In prolonged reactions (up to 20h) a spot appeared on the thin-layer plates, which had the same colour and R_F value as phenylalanine. However, no firm evidence for the liberation of free amino acids from bradykinin by enzyme II was obtained. The effect of 0.2M-NaCl on these reactions was not similar to that observed with the derivatives of 2-naphthylamine: no noticeable activation was found. *p*-Chloromercuribenzoate did not have any detectable effect on the action of enzyme II on bradykinin. In prolonged reactions at 30°C spontaneous hydrolysis of bradykinin was found, but the enzyme-catalysed reactions were remarkably faster. The bradykinin spot (purple or violet) had R_F 0.08–0.12. The spot with R_F 0.39–0.43 was yellow, but no free proline was found with certainty.

The following peptides were clearly hydrolysed by enzyme II: Leu-Leu (0.2M-NaCl slightly inhibited this reaction), Leu-Gly and Pro-Phe-NH_2. No detectable hydrolysis under the conditions involved was observed in 60min with the following peptides:



Gly-Gly, Gly-DL-Ala, Pro-Gly, Gly-Ile, hippuryl-proline, Gly-Pro-Gly-Gly, poly-(L-proline), poly-(L-hydroxyproline) and poly-(L-lysine). Enzyme II did not cause detectable hydrolysis of denatured haemoglobin.

Affinity chromatography on amino acid–Sepharose 4B gels. The nature of the binding of amino acid 2-naphthylamide substrates to enzyme II was studied by using affinity chromatography with columns packed with Sepharose 4B gel covalently coupled to various amino acids. Portions (50μl) of the enzyme from step 9 were diluted with 0.5ml of the elution buffer and the 0.55ml samples were applied on identical columns (25cm × 1cm) packed with the gels mentioned. The 50μl sample was sufficiently small to bind as a whole to L-arginine–Sepharose 4B in these conditions: no enzyme could be eluted from the column by using the buffer alone (Fig. 6*a*). The addition of 1.0M-NaCl to the elution buffer detached the enzyme. Fig. 6(*b*) shows that L-lysine–Sepharose 4B did not bind the enzyme in its entirety; small amounts of enzyme could be eluted with the buffer alone. L-Ornithine–Sepharose 4B bound a still smaller amount of the enzyme (Fig. 6*c*). The ability of the enzyme to bind to the amino acid-substituted gels thus seemed to depend on the length of the side chain of the basic amino acid involved. The binding also correlated with the differences in the rates of hydrolysis of the three corresponding naphthylamides: the arginine derivative was hydrolysed rapidly, the lysine derivative slowly, and the ornithine derivative was not hydrolysed at all. Figs. 6(*d*)–(*g*) show that the enzyme was not bound to any significant extent to Sepharose 4B covalently coupled to L-proline, L-methionine, L-leucine or DL-phenylalanine. However, the enzyme hydrolysed the corre-

Fig. 6. *Affinity chromatography (descending) of enzyme II*
The column dimensions were 1.0cm × 25cm (19.6cm^3) and the column was packed with Sepharose 4B coupled to various amino acids with elution buffer 0.01M-3,3-dimethylglutarate buffer, pH7.2, or the same buffer containing 1.0M-NaCl. The hydrostatic pressure was 10–12cm (temperature 4°C) and fractions of volume 0.5ml were collected at a flow rate of 0.35ml/min. Samples (50μl) of enzyme from step 9 were applied to the column. (*a*) L-Arginine–Sepharose 4B; (*b*) L-lysine–Sepharose 4B; (*c*) L-ornithine–Sepharose 4B; (*d*) L-leucine–Sepharose 4B; (*e*) L-methionine–Sepharose 4B; (*f*) DL-phenylalanine–Sepharose 4B; (*g*) L-proline–Sepharose 4B; (*h*) unsubstituted Sepharose 4B. The columns were eluted up to fraction 50 with buffer that did not contain added NaCl, and thereafter with the same buffer containing 1.0M-NaCl (buffer change indicated by arrow). The enzymic activities are in arbitrary units, as reaction times of various length (up to 16h) were used to reveal small enzyme peaks.

sponding amino acid 2-naphthylamides at a considerable rate (Table 3). If very small amounts of the enzyme were bound to these gels they were not de-adsorbable with 1M-NaCl. Indeed, very weak binding may have occurred to Sepharose 4B coupled to L-methionine and L-proline, because L-methionine- and L-proline-Sepharose columns produced broader enzyme peaks and slightly lower recovery of the enzyme activity than did the other gels. The elution profiles of the enzyme generally depended on the nature of the amino acid coupled to Sepharose. Fig. 6(*h*) shows that the enzyme was not bound to unsubstituted Sepharose 4B. As small samples of diluted enzyme were deliberately applied to the columns, the determination of proteins in the fractions was not conceivable. Figs. 6(*d*), 6(*e*), 6(*g*) and 6(*h*) showed some heterogeneity.

Effect of inorganic ions. Dialysed preparations of enzymes I and II were tested in the presence of several bivalent metal cations. The activity of enzyme I was not affected to any remarkable extent by Mg^{2+}, Ba^{2+}, Sr^{2+}, or Mn^{2+}, whereas Ca^{2+}, Cu^{2+} and Zn^{2+} were inhibitory (Fig. 7). Ba^{2+} ions produced turbidity, observable with difficulty, which was removed by centrifugation for 10min at 23500$g_{av.}$ before spectrophotometric measurements. Omission of centrifugation was found to lead to increased absorbance values and a false idea about the effects of Ba^{2+} on the rates of the hydrolysis. The results were almost similar when the effects of the above metal cations were tested with the arginine and alanine derivatives of 2-naphthylamine.

The activity of the Cl^--dependent enzyme II, tested in the presence of 0.2M-NaCl, was not affected to any noticeable extent by Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Co^{2+} (Fig. 8). However, other metals of the fourth period, with lower (Mn) or higher (Cu and Zn) atomic number than cobalt, were inhibitory. The results shown in Fig. 8 were obtained with L-arginine 2-naphthylamide, but the effects of the above metal cations were similar when the alanine derivative was tested in the presence of 0.2M-NaCl.

Enzyme I was specifically activated by Co^{2+} (Fig. 9). The curves of rate against concentration of Co^{2+} reached a maximum at 0.5mM-metal-ion concentration, but with substrates containing a basic amino acid the maximum was broader (0.2–0.5mM) than with other substrates. The red colour of the Co^{2+} in the reaction mixtures was taken into account with appropriate controls. EDTA (disodium salt) slightly inhibited the Co^{2+}-dependent enzyme I and the degree of inhibition was dependent on the substrate used (Fig. 10). Enzyme II, tested in a similar experiment with L-alanine and L-arginine 2-naphthylamides (in the presence of 0.2M-NaCl), was not affected by EDTA.

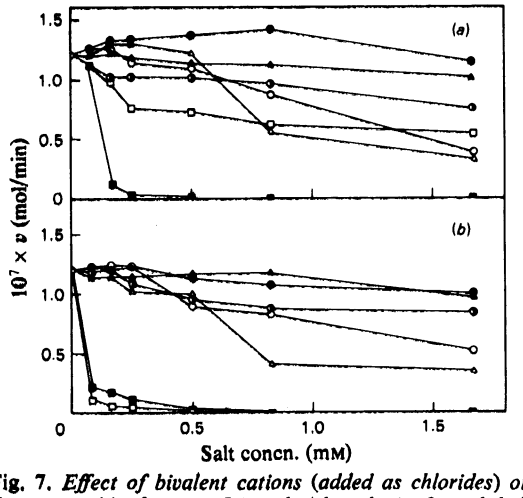


Fig. 7. *Effect of bivalent cations (added as chlorides) on the activity (v) of enzyme I tested with L-alanine 2-naphthylamide (a) and L-arginine 2-naphthylamide (b)*
The reactions were carried out in 0.05M-phosphate buffer, pH7.0. Symbols: ○, Ba^{2+}; ●, Sr^{2+}; △, Ca^{2+}; ▲, Mg^{2+}; □, Zn^{2+}; ■, Cu^{2+}, ◑, Mn^{2+}.

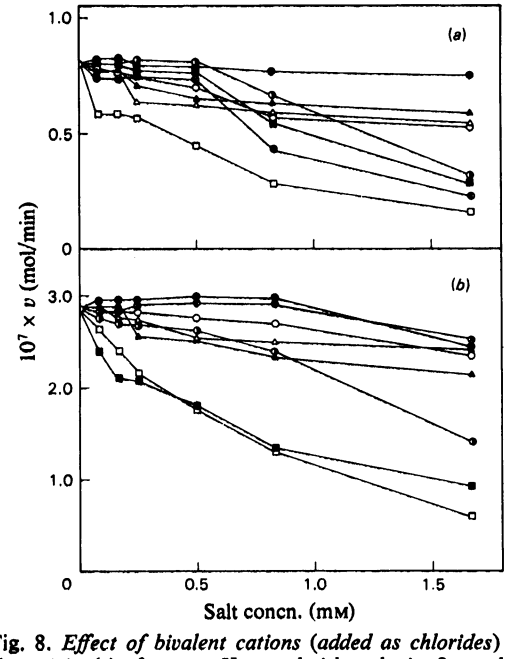


Fig. 8. *Effect of bivalent cations (added as chlorides) on the activity (v) of enzyme II, tested with L-alanine 2-napthylamide (a) and L-arginine 2-naphthylamide (b)*
The reactions were carried out in 0.05M-phosphate buffer, pH6.2, which contained 0.2M-NaCl. Symbols: ○, Ba^{2+}; ●, Sr^{2+}; △, Ca^{2+}; ▲, Mg^{2+}; □, Zn^{2+}; ■, Cu^{2+}; ◑, Mn^{2+}; ⊗, Co^{2+}.

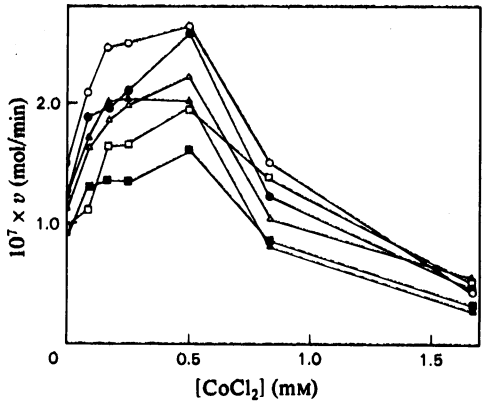


Fig. 9. *Effect of $CoCl_2$ concentration on the activity of enzyme I*

The reactions were carried out in 0.05M-phosphate buffer, pH7.0. The amino acid 2-naphthylamides (2-NNap) were: ○, lysine 2-NNap; ●, alanine 2-NNap; △, methionine 2-NNap; ▲, arginine 2-NNap; □, leucine 2-NNap; ■, phenylalanine 2-NNap.

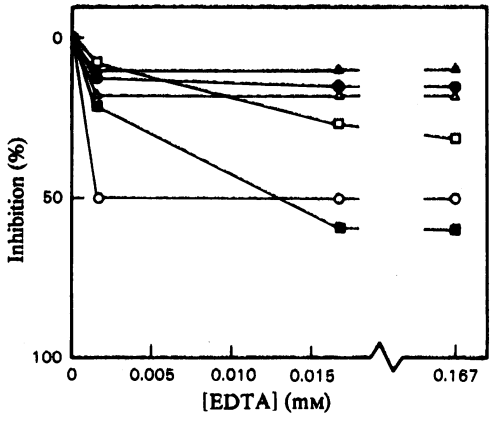


Fig. 10. *Effect of EDTA concentration on the activity of enzyme I*

The reactions were carried out in 0.05M-phosphate buffer, pH7.0, without added Co^{2+} ions. The amino acid 2-naphthylamides (2-NNap) were: ○, alanine 2-NNap; ●, arginine 2-NNap; △, leucine 2-NNap; ▲, lysine 2-NNap; ■, methionine 2-NNap; □, phenylalanine 2-NNap.

An important property of mammalian and avian arginine aminopeptidases is the dependence of the enzyme activity on Cl^- (Mäkinen, 1975). The two erythrocyte enzymes clearly differed from each other in this (Fig. 11). The pH-dependence of the chloride effect is shown in Fig. 12 for both enzymes. NaCl did not affect the pH-dependence curve of enzyme I.

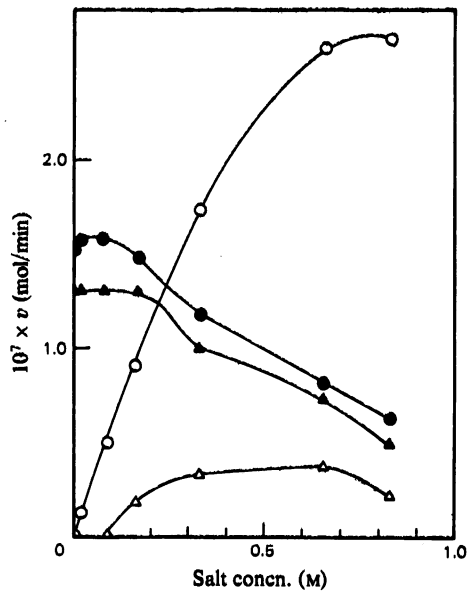


Fig. 11. *Effect of concentration of NaCl and KF on the activity of enzymes I and II*

The reactions were carried out in 0.05M-phosphate buffer, pH7.2. The substrate was L-arginine 2-naphthylamide. Symbols: ●, enzyme I and NaCl; ▲, enzyme I and KF; ○, enzyme II and NaCl; △, enzyme II and KF.

Virtually no activity was observed with enzyme II in the absence of Cl^-. Fig. 13 shows that the form of the curves for rate against concentration of NaCl with enzyme II greatly depended on the nature of the amino acid side chain. The effect of F^-, determined at pH7.2, when most inorganic fluoride in an aqueous solution is in the form of ions, is shown in Fig. 11. The activity of enzyme I first decreased at rather high fluoride concentrations (0.33M), and enzyme II was inactive at low and active at higher concentrations.

Bicarbonate (0.0083–0.166M), tested at pH7.20–7.33 in 0.5M-3,3-dimethylglutarate buffer with no added Cl^-, did not lead to any measurable rate of hydrolysis of L-arginine 2-naphthylamide or L-alanine 2-naphthylamide by enzyme II. However, because enzyme I does not require Cl^- for maximum activity, it was active in the presence of 0.001–0.1M-bicarbonate at pH7.20–7.33 in the above buffer when tested with both of the two substrates mentioned. The activity was practically constant between 0.008 and 0.04M-bicarbonate concentrations. Higher amounts were gradually inhibitory. Thus 0.083M-bicarbonate inhibited by 20%, 0.125M-bicarbonate by 44% and 0.166M-bicarbonate by 60%. In the experiments concerning the effect of bicarbonate the pH of the reaction mixtures was controlled with a

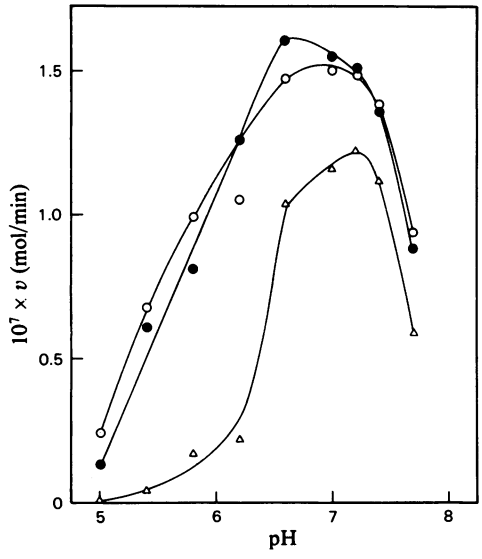


Fig. 12. *Effect of pH on the activity of enzymes I and II in the presence of* 0.2M-*NaCl and without added salt*
Assays were carried out in 0.05M-3,3-dimethylglutarate buffer. The substrate was L-arginine 2-naphthylamide. Symbols: ●, enzyme I without added salt; ○, enzyme I in 0.2M-NaCl; △, enzyme II in 0.2M-NaCl.

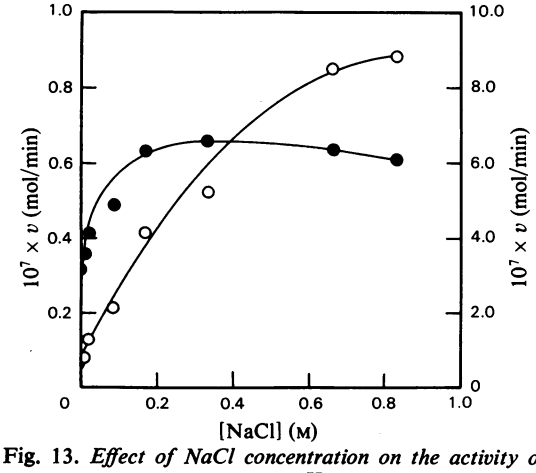


Fig. 13. *Effect of NaCl concentration on the activity of enzyme II*
Assays were carried out in 0.05M-phosphate buffer, pH7.2. Symbols: ○, L-lysine 2-naphthylamide (left-hand scale); ●, L-alanine 2-naphthylamide (right-hand scale).

combination electrode. The bicarbonate/3,3-dimethylglutarate buffers were freshly prepared and their total bicarbonate and CO_2 content, determined with a carbon dioxide electrode (Orion Research Incorporated, Cambridge, MA, U.S.A.), corresponded to the amounts of $NaHCO_3$ added. The CO_2 pressure of the reaction mixtures was not determined.

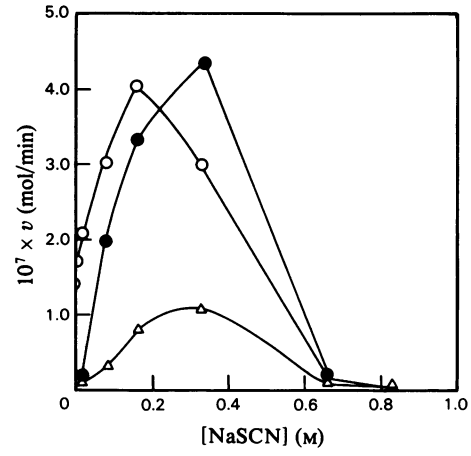


Fig. 14. *Effect of NaSCN concentration on the activity of enzyme II*
Assays were carried out in 0.05M-phosphate buffer, pH7.2, without added NaCl. Symbols: ○, L-alanine 2-naphthylamide; ●, L-arginine 2-naphthylamide; △, L-lysine 2-naphthylamide.

The effect of SCN^- on the activity of enzyme II is shown in Fig. 14 with three substrates. Both substrates with a basic side chain had the maximum rate of hydrolysis at 0.33M-NaSCN. At zero concentration of NaSCN the enzyme activity towards these substrates was practically nil, whereas L-alanine 2-naphthylamide was clearly hydrolysed. These experiments were carried out without added NaCl. Consequently, SCN^- could partly replace Cl^- in the hydrolysis reactions catalysed by enzyme II.

Product inhibition. L-Arginine and L-lysine tested between concentrations of 0.125mM and 1.0mM did not have any effect on the rate of the hydrolysis of L-arginine 2-naphthylamide catalysed by enzyme II. These experiments were carried out in 0.025M-phosphate buffer, pH7.2, in the presence of 0.2M-NaCl. ε-Amino-n-hexanoic acid (1.67mM) did not affect the above reaction either.

Determination of K_m values. Fig. 15 shows typical Hanes' plots for the hydrolysis of L-arginine 2-naphthylamide catalysed by enzymes I and II. The values of K_m and $V_{app.}$, calculated from the plots at the pH values indicated, were similar to those obtained by the Eisenthal & Cornish-Bowden (1974) method shown in Table 5 for the same pH values.

A characteristic of the Cl^--dependent aminopeptidase B of rat liver specifically acting on L-arginine 2-naphthylamide and L-lysine 2-naphthylamide was shown to be a rather strong substrate

inhibition at alkaline pH values (Mäkinen, 1969; Mäkinen & Mäkinen, 1972). The effect of the substrate (arginine derivative) concentration on the rate of the hydrolysis catalysed by enzymes I and II was studied at 21 different substrate concentrations comprising a wide range (0.833 μM–0.833 mM) in three different buffers (shown in Fig. 15), each comprising nine different pH values. The data showed slight or no substrate inhibition at alkaline pH values with enzyme II, but enzyme I showed clear substrate inhibition (Fig. 16).

Effect of p-chloromercuribenzoate. The activity of enzyme II was tested in the presence of 0.167 μM–0.167 mM-*p*-chloromercuribenzoate (sodium salt), with several L-amino acid 2-naphthylamides as substrates (those that were hydrolysed by enzyme II

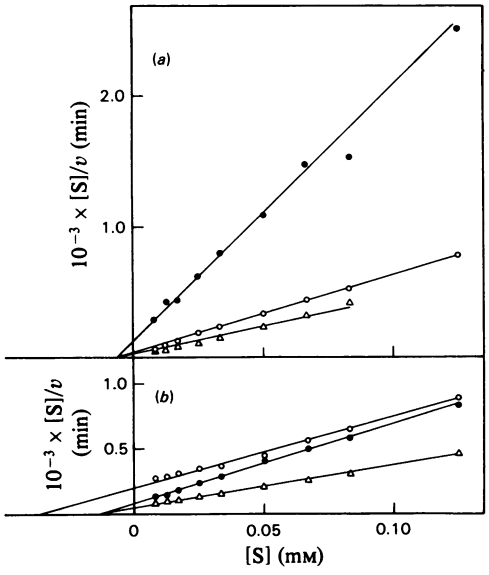


Fig. 15. *Determination of K_m values of enzymes I and II*
(*a*) Hanes plot of substrate concentration divided by the initial velocity against the substrate concentration in the hydrolysis of L-arginine 2-naphthylamide catalysed by enzyme II, and determined in the presence of 0.2 M-NaCl. (*b*) The same for enzyme I (no added NaCl). Symbols: ●, 0.025 M-phosphate buffer, pH7.0; ○, 0.025 M-3,3-dimethylglutarate buffer, pH7.0; △, 0.01 M-borate buffer, pH7.3.

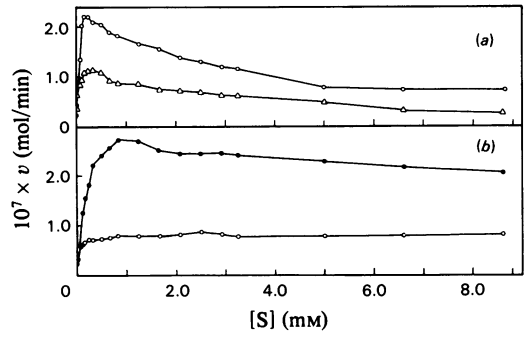


Fig. 16. *Effect of high substrate concentrations on the activity of enzymes I and II*
(*a*) Michaelis–Menten plot of initial velocity against the substrate concentration in the hydrolysis of L-arginine 2-naphthylamide catalysed by enzyme I. The buffer was 0.01 M-borate buffer, pH8.2 (○) or pH8.8 (△), determined without added Cl^-. (*b*) The same conditions for enzyme II. The buffer was 0.01 M-borate buffer, pH8.2 (○) or pH7.3 (●), and the reactions were carried out in the presence of 0.2 M-NaCl.

Table 5. *K_m and $V_{app.}$ values for enzyme II*
The concentration of L-arginine 2-naphthylamide was varied from 12.5 μM to 166.7 μM * or from 0.83 μM to 25 μM †, and the assay temperature was 30°C. The values were obtained from the plots described by Eisenthal & Cornish-Bowden (1974). Details of Buffers are given in the Materials and Methods section.

Buffer	pH	K_m (μM)	$10^7 \times V_{app.}$ (mol/min)
25 mM-Phosphate	6.8	4.6	1.09†
25 mM-Phosphate	7.0	7.4	1.23†
25 mM-Phosphate	7.2	8.0	1.45†
25 mM-Phosphate	7.4	11.5	1.42†
25 mM-Phosphate	7.6	6.0	1.05†
25 mM-Phosphate	7.8	5.7	0.83†
25 mM-Phosphate	8.0	3.1	0.51†
25 mM-3,3-Dimethylglutarate	7.4	10.0	1.37*
25 mM-3,3-Dimethylglutarate	7.7	12.5	0.78*
10 mM-Borate	7.3	13.0	2.87*
10 mM-Borate	7.6	9.5	2.21*
10 mM-Borate	7.8	4.0	1.79*
10 mM-Borate	8.0	4.5	0.88*

in the presence of 0.2M-NaCl; Table 4). The inhibition was in all cases of the order of 10–20% only at the highest inhibitor concentrations used.

Discussion

The original aim of the present study was to investigate human erythrocyte enzymes resembling the aminopeptidase B of rat liver (EC 3.4.11.6). Because aminopeptidase B specifically hydrolyses *N*-terminal arginyl and lysyl residues only, L-arginine 2-naphthylamide was used as the working substrate. The presence of aminopeptidase B and similar Cl^--dependent enzymes in biological fluids has been demonstrated by carrying out the enzyme assays simultaneously in the presence and absence of 0.2M-NaCl; the presence of NaCl in the reaction mixtures has previously revealed the enzyme after molecular-permeation chromatography (Mäkinen & Oksala, 1973; Mäkinen & Virtanen, 1976). The use of 0.2M-Cl^- in reaction mixtures, when aminopeptidase B may have its maximum activity at 0.154M-NaCl (0.9%), was intended to mask unspecific and less-important salt effects on the enzyme. The use of this NaCl criterion in the present study revealed in erythrocytes a Cl^--dependent arylamidase (enzyme II) and another enzyme that was slightly inhibited by NaCl (enzyme I). The dependence of enzyme II on Cl^- was not absolute, as low activity with some substrates (2-naphthylamide derivatives of leucine, methionine and valine) was observed without added salt, but the hydrolysis of basic amino acid 2-naphthylamides clearly required the presence of Cl^-.

Several characteristics of enzyme II clearly differed from those of aminopeptidase B of rat liver. The most noticeable differences were that aminopeptidase B was strongly inhibited by *p*-chloromercuribenzoate, whereas enzyme II was not affected by this compound. The plots of the rate against the concentration of NaCl for these enzymes also have a different form: with aminopeptidase B a maximum at 0.15–0.2M-NaCl was observed, but no maximum was found with enzyme II up to 0.83M-NaCl. Perhaps the most important difference was, however, the substrate specificity: the rat liver enzyme was very specific, whereas enzyme II hydrolysed substrates with very diverse amino acid side chains. The most important similarity of enzyme II and aminopeptidase B was thus confined to the activation by certain Cl^- concentrations in the hydrolysis of L-arginine 2-naphthylamide. Although the absence of a strict specificity contrasts with the rat liver enzyme, it may be justified to use the term aminopeptidase B for enzyme II, if this general designation is reserved for peptidohydrolases showing preference for *N*-terminal lysine or arginine residues.

Human blood-cell aminopeptidases were studied with L-alanine 2-naphthylamide by Neef *et al.* (1973). The highest aminopeptidase activity was found in the granulocytes, the erythrocytes displaying approx. 50% lower activity. Oxytocinases (cystine aminopeptidases) have been studied by Yman (1970), Klimek (1968) and Klimek & Malolepszy (1968) in human blood and serum. Aminopeptidase A (Nagatsu *et al.*, 1970) and angiotensinase (an aminopeptidase) were studied in plasma (Kurtz & Wachsmuth, 1969), and several other aminopeptidases were demonstrated in platelets (Greiff *et al.*, 1969). These enzymes have been studied with aminoacyl derivatives of either 2-naphthylamine or 4-nitroaniline as substrates. The present results warrant naming enzymes capable of rapidly hydrolysing these substrates aminopeptidases, in the absence of more detailed studies of specificity on peptides. For example, enzyme II not only hydrolysed synthetic chromogenic substrates, but also hydrolysed bradykinin at internal peptide bonds, and attacked substrates (hippuryl derivatives) with blocked α-amino function. In the latter cases the enzyme may, however, bind to the basic side chains of hippurylarginine and hippuryl-lysine, suggesting that the specificity requirements of enzyme II may be confined to the presence of one NH_3^+ group (either α-amino or side chain), or one imino group (proline), in the peptide substrate. Enzyme II may also be regarded as an endopeptidase, if the hydrolysis of bradykinin is also used as a criterion. More detailed specificity studies on enzyme II are needed in this respect.

Other aryiaminopeptidases recently studied include an enzyme from rat cardiac-muscle extracts, which hydrolysed L-lysine 4-nitroanilide (Bury *et al.*, 1977). The characteristics reported by Bury and his co-workers for their enzyme II resembled very much those of rat liver aminopeptidase B, which has also been demonstrated in rat heart (Mäkinen & Hopsu-Havu, 1967). Garner & Behal (1977) described a human liver alanine aminopeptidase that was inhibited by NaCl. The inhibitory effect of 0.2M-NaCl on the hydrolysis of L-alanine 2-naphthylamide has been previously shown (Mäkinen *et al.*, 1970), and it is very likely that the liver tissue studied by Garner & Behal (1977) also contained an erythrocyte enzyme similar to enzyme I.

L-Ornithine 2-naphthylamide competitively inhibits the hydrolysis of the corresponding arginine and lysine derivatives catalysed by rat liver aminopeptidase B (Mäkinen, 1975). The ornithine derivative is bound to the active site, but its hydrolysis does not take place. Because the values of pK_3 of the amino acids concerned are of approximately the same magnitude (12.5 for arginine, 10.79 for lysine and 10.76 for ornithine, at 25°C), the unsuitability of the ornithine derivative as substrate for aminopeptidase B and enzymes I and II described in the present paper could be explained in terms of differences in the

lengths of the amino acid side chains involved. On the basis of these considerations, an induced-fit mechanism was suggested to determine the substrate specificity of rat liver aminopeptidase B (Mäkinen, 1975). In such a case the shorter side chain of the ornithine residue would not be able to trigger the necessary reactions at the active site after binding. This mechanism would also partly explain why the arginine derivative with its 'bulkier' $-NHC(NH_2)_2^+$ group is a better substrate than the lysine derivative for aminopeptidase B and enzyme II.

Anionic activation of enzymes was previously considered rare, and when these types of effect occurred, they were often termed unspecific. The present line of research pursued in our laboratories has particularly attempted to elucidate the Cl^- effect of aminopeptidases. The accumulated information suggests that aminopeptidase B and enzyme II of the present study, and related peptidohydrolases studied in our laboratories, are affected by Cl^- at the physiological concentration (0.9% or 0.154M). These enzymes probably participate in inflammatory reactions, as suggested by a number of studies (Hopsu-Havu *et al.*, 1966; Mäkinen, 1975; Mäkinen & Paunio, 1972; Mäkinen & Oksala, 1973; Mäkinen & Virtanen, 1976; Virtanen & Mäkinen, 1974; Mäkinen & Hyyppä, 1975; Mäkinen *et al.*, 1975; Virtanen *et al.*, 1977). This point of view is also supported by recent findings about the occurrence in leucocytes of enzymes resembling aminopeptidase B and enzyme II (Söderling *et al.*, 1977; Knuuttila *et al.*, 1978). The environments in which these enzymes have been suggested to function, namely blood plasma, interstitial fluid, inflammatory exudates and erythrocytes, are all characterized by a high concentration of Cl^-. The leucocyte enzymes were suggested to take part in the metabolism of inflammatory peptides.

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