Isolation of rat submandibular kallikreins by using immunoabsorption chromatography

Kaare M. GAUTVIK,* Liv JOHANSEN,† Kirsten SVINDAHL,* Kjell NUSTAD* and
Torill B. ØRSTAVIK†

*The Norwegian Radium Hospital, Oslo, Norway, and †Institute of Physiology and Biochemistry,
Dental Faculty, University of Oslo, Norway

(Received 13 August 1979)

A one-step immunoabsorption method for the isolation of glandular kallikreins is described using the immunoglobulin fraction from rabbit anti-(rat glandular kallikrein) serum coupled to CNBr-activated Sepharose 4B. The adsorptions of ^{125}I -labelled kallikrein or unlabelled kallikrein from 100 000 g submandibular gland supernatants were more than 97% complete. The elution of kallikrein from the immunoabsorbent using guanidine hydrochloride gave about 20% yield, which could be increased up to 70% by including 0.5% bovine serum albumin in the elution buffer. The electrophoretic mobility of eluted submandibular ^{125}I -labelled kallikrein or submandibular glandular kallikrein was not altered after affinity chromatography, as judged by conventional polyacrylamide disc-gel electrophoresis or by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. In addition, the specific esterase and the kininogenase activities of isolated submandibular kallikreins were more than 90% of those of the reference enzyme. This procedure, which results in the isolation of immunologically and biologically active submandibular kallikrein, may also be used for purification of other glandular kallikreins that show immunological homology.

Glandular kallikreins (EC 3.4.21.8) are a group of related serine proteinases. They are present in exocrine glands and kidney, as well as in their secretions. These enzymes have a very restricted substrate specificity, and their main function is probably related to generation of kinins from specific precursors in plasma and interstitial fluid. Kinins are peptides with potent biological actions directed towards smooth muscles and possibly towards transporting epithelia (Gautvik *et al.*, 1976a,b; Nustad *et al.*, 1978). Glandular kallikreins from different organs and animals have been obtained in highly purified forms by using a combination of ion-exchange chromatography, hydroxyapatite chromatography, isoelectric focusing and gel filtration (Nustad & Pierce, 1974; Nustad *et al.*, 1978). Alternatively, affinity chromatography using kallikrein-inhibitor, substrate or anti-kallikrein antibody has also been used (Nustad & Pierce, 1974; Nustad *et al.*, 1978).

In the present study we describe a one-step purification procedure employing immunoabsorption chromatography that results in a high yield of the kallikrein preparations. The isolated rat submandibular kallikrein showed good preservation of enzymatic and immunological activity.

Experimental

Production of anti-(rat glandular kallikrein) serum

A highly purified preparation of rat submandibular gland kallikrein was used as the immunogen (Brandtzaeg *et al.*, 1976). Enzyme protein (100–300 μg as determined by the method of Lowry *et al.* (1951) was mixed with an equal volume of incomplete or complete Freund's adjuvant (DifCo) and injected into multiple intracutaneous sites in rabbits. The animals were bled 10 days after each injection, and serum from the different bleedings was tested for antibody formation (Nustad *et al.*, 1979). The following experiments were carried out using antiserum produced from one rabbit.

Preparation of the immunosorbent

The immunoglobulin G fraction was isolated as described previously (Brandtzaeg *et al.*, 1976). Briefly, 5 ml of serum was mixed with DEAE-Sephadex A-50 (5 g wet wt.), pre-equilibrated with 0.1 M-Tris/HCl buffer (pH 8.0), and the mixture was stirred for 1 h at 4°C. After filtration, the gel was washed with 5 ml of the buffer. The adsorption procedure was repeated, and the combined filtrates (about 15 ml) were precipitated with $(\text{NH}_4)_2\text{SO}_4$.

(50% saturation) with stirring for 18 h at 4°C. After centrifugation (10000 g, 10 min), the precipitate was dissolved in 1.2 ml of distilled water, and dialysed for 2–3 days against phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.4), centrifuged (500 g, 10 min), and the purity of the immunoglobulin G fraction in the supernatant was analysed by immunoelectrophoresis against sheep anti-(rabbit immunoglobulin) serum. CNBr-activated Sepharose 4B (Pharmacia) was equilibrated in and washed with 1 mM-HCl. The prepared gel was mixed with a portion of the immunoglobulin G fraction [10 mg of protein determined by the method of Lowry *et al.* (1951)] in 5 ml of 0.1 M-NaHCO₃/0.5 M-NaCl, pH 9.5 and the mixture was shaken for 2 h at 18°C. The gel was then washed on a glass filter with phosphate-buffered saline, and the remaining active groups were neutralized with 10 ml of 1 M-ethanolamine (pH 8.0) for 1 h with rotation. After washing the gel once with 1.0 M-sodium acetate/1.0 M-NaCl buffer, pH 4, and three times with 0.1 M-sodium borate/1.0 M-NaCl buffer, pH 8, it was stored at 4°C in phosphate-buffered saline containing 0.01 M-merthiolate. The efficiency of coupling as judged by the recovery of proteins (A_{280} , in eluates and in washings was 90% or better.

Iodination of rat submandibular gland kallikreins

A purified preparation of rat submandibular gland kallikreins (Brandtzaeg *et al.*, 1976) was labelled with Na¹²⁵I (The Radiochemical Centre, Amersham, Bucks., U.K.) by using hypochlorite as oxidizing agent. The iodination was carried out in 0.5 M-sodium phosphate buffer, pH 7.5, with 0.5 mCi of ¹²⁵I (5 µl), rat submandibular kallikrein, (24 µg of protein, 4 µl), NaClO (10 µl of 0.125 M), and 500 µl of 0.05 M-NaHPO₄ containing 0.5 mg of Na₂S₂O₅, 0.1% bovine serum albumin, and 5 mg of KI. The time interval between addition of the oxidizing agent and the reducing agent was about 10 s. Removal of free ¹²⁵I- was carried out by gel filtration on Sephadex G-75 (Pharmacia) pre-equilibrated with 0.15 M-NaCl containing 0.1% bovine serum albumin. The iodinated kallikrein, which was collected in 0.5-ml fractions, was eluted as one peak. The iodinated kallikrein preparation, which retained full immunological and enzymatic activity, (Ørstavik *et al.*, 1980) was pooled and stored in 10–20 µl samples at –20°C for later use.

Measurements of kallikrein activities

The enzyme was quantified by its esterase activity, using as substrate α -N-benzoyl-arginine ethyl ester hydrochloride (Nustad *et al.*, 1975). One esterase unit was defined as the amount of enzyme hydrolysing one µmol of substrate/min. The kinin-forming activity was also measured using heated (56°C, 30 min) dog plasma as a source of kininogen

in the presence of phenanthroline (30 mM). The oestrogen-primed rat uterus preparation was used for bioassay (Gallimore *et al.*, 1978).

Immunoelectrophoresis of rabbit anti-(rat glandular kallikrein) immunoglobulin G fraction

Electrophoresis was carried out in agarose gel (Litex) (1.5%, w/v) dissolved in 0.075 M-veronal/calcium lactate buffer (pH 6.8) for 45 min, using 140 V. After application of sheep anti-(rabbit immunoglobulin) serum (Dakopatt, DAKO-Immunoglobulins, Copenhagen, Denmark) the immunoprecipitation was allowed to proceed for 18 h at 4°C.

Polyacrylamide-gel electrophoresis

Gel electrophoresis with and without sodium dodecyl sulphate was carried out as described previously (Gautvik *et al.*, 1976b).

Purification of rat glandular kallikrein by immuno-adsorption

Different exocrine organs from the rat (submandibular glands, exorbital lacrimal glands and pancreas) were homogenized in phosphate-buffered saline (wet wt./vol. 1:10) using 30 strokes of a Potter–Elvehjem homogenizer at 4°C; the homogenates were centrifuged (100000 g, 10 min, 4°C). The supernatants were collected and used for adsorption studies. The amount of glandular kallikrein in the homogenates was determined radio-immunologically (Nustad *et al.*, 1979) before application to the immuno-adsorption column (1 ml of gel).

After washing the column in phosphate-buffered saline, the kallikrein was eluted by guanidine hydrochloride (4 M or 8 M) (Schwartz–Mann), with or without bovine serum albumin, at room temperature (20°C).

Radioimmunoassay of rat glandular kallikreins

The protocol described by Nustad *et al.* (1979) was used. Briefly, the incubations were carried out in 0.05 M-barbital buffer/0.1% bovine serum albumin/0.01% merthiolate (pH 8.6) containing 100 µl of rat submandibular ¹²⁵I-labelled kallikrein solution (20000 c.p.m., 0.2 ng), 100 µl of antiserum (1:80000 final dilution) and 200 µl of sample (rat submandibular, rat pancreatic or rat lacrimal kallikrein preparations). Free and bound ¹²⁵I-labelled-kallikrein were separated by using double-antibody solid phase (DASP; Organon, Oss, The Netherlands).

Results

Immunoreactivity of the isolated immunoglobulin G fraction from the rabbit anti-(rat submandibular gland kallikrein) serum

When the isolated rabbit immunoglobulin frac-

tion from rabbit anti-kallikrein serum was tested against sheep anti-(rabbit immunoglobulin) serum, a major precipitation line for immunoglobulin was seen after immunoelectrophoresis. As well as immunoglobulin G, an additional line, possibly representing transferrin, was observed. The partly purified immunoglobulin G fraction bound ^{125}I -labelled kallikrein in a manner entirely analogous to that of the antikallikrein serum (data not shown). Thus, the procedure for the isolation of the immunoglobulin fraction did not affect its ability to bind ^{125}I -labelled kallikrein.

Immunoabsorption of glandular ^{125}I -labelled kallikrein on CNBr-activated Sepharose 4B and its subsequent elution

The isolated immunoglobulin fraction from the

Fig. 1. Immunoabsorption of ^{125}I -labelled kallikrein (7.5×10^5 c.p.m.) to Sepharose 4B coupled to antikallikrein immunoglobulin G fraction as described in the Experimental section

After rotation for 1 h, the gel mixture (1 ml) was applied to a column (1 cm $\times$ 3 cm) and the eluate was collected for radioactivity measurements. The column was washed with 15 ml of phosphate-buffered saline and ^{125}I -labelled kallikrein was eluted with 4 M-guanidine hydrochloride (see the Experimental section). The radioactivity in the different fractions is shown. The arrow shows the application of the eluent.

rabbit antiserum was coupled to activated Sepharose 4B as described in the Experimental section. The iodinated submandibular enzyme was mixed in batches with the prepared immunoabsorbent material, transferred to a short column with gel volume of 1 ml and washed with buffer as outlined in the Experimental section. Fig. 1 shows that some radioactivity passed through the gel after the first washings. However, during the subsequent washings, no radioactivity was recovered. Immediately after the start of the addition of 4 M-guanidine hydrochloride to the gel, there was a sharp increase in eluted radioactivity, which again declines rapidly in the subsequent fractions.

Immunoabsorption of submandibular ^{125}I -labelled kallikrein and characterization of the radioactive, eluted material by electrophoresis

Fig. 2 shows conventional disc-gel electrophoresis [16% (w/v) polyacrylamide gel] of submandibular ^{125}I -labelled kallikrein before and after affinity chromatography. For comparison, an unlabelled reference kallikrein preparation was electrophoresed on parallel gels and the mobility was measured after Coomassie Blue staining (Gautvik *et al.*, 1976b). The radioactive material eluted from the immunoabsorption column showed the same mobility in disc-gel electrophoresis as the rat submandibular ^{125}I -labelled kallikreins. The reference enzyme preparation, which contains multiple forms of the enzyme (Nustad & Pierce, 1974; Brandtzaeg *et al.*, 1976), will, on prolonged storage, give rise to small amounts of aggregation and degradation products (Fig. 2). Immunoabsorption

Fig. 2. Disc-gel electrophoresis [16% (w/v) polyacrylamide gel] of ^{125}I -labelled rat submandibular kallikrein before (●) and after (○) affinity chromatography. Purified rat submandibular kallikrein was used as reference (upper part). The affinity chromatography procedure and disc gel electrophoresis were carried out as described in the Experimental section.

and elution by guanidine hydrochloride of the radioactive-labelled kallikrein caused no change in the electrophoretic mobility of the enzyme.

To test the stability of ^{125}I -labelled kallikrein added to homogenates of submandibular glands, which contain proteolytic activity, polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out (see the Experimental section). Radioactive-labelled enzyme was mixed with glandular homogenate and incubated at room temperature for 30 min; aliquots were then subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in comparison with non-incubated iodinated enzyme as well as the reference glandular kallikrein. Fig. 3 shows that the radioactive peaks, as well as endogenous submandibular kallikrein isolated by affinity chromatography (position marked on the abscissa), have an identical electrophoretic mobility. No radioactive fragments could be demonstrated after incubation with the gland homogenate.

To test possible non-biospecific interactions between the antigen and the solid phase the following

Fig. 3. Stability and electrophoretic mobility of endogenous kallikrein

A mixture of rat submandibular gland supernatant (see the Experimental section) and ^{125}I -labelled rat submandibular kallikrein was submitted to immuno-adsorption chromatography to test the stability of ^{125}I -labelled kallikrein in gland homogenate and the electrophoretic mobility of endogenously isolated kallikrein. Polyacrylamide gel (7.5%, w/v) electrophoresis in sodium dodecyl sulphate is shown. The two radioactive kallikrein preparations (●, before and O, after chromatography), and the endogenous submandibular kallikrein isolated by affinity chromatography (≡) (detected by Coomassie Blue staining) had the same electrophoretic mobility as a reference kallikrein preparation (not shown).

control experiments were carried out. (1) Inactivated Sepharose 4B was rotated with ^{125}I -labelled kallikrein and more than 90% of the activity was recovered in the first washing. (2) Non-immune-rabbit immunoglobulin G was coupled to activated Sepharose 4B with a coupling efficiency of 93%. After inactivation, the Sepharose was incubated with ^{125}I -labelled kallikrein; more than 80% of the activity did not bind to the solid phase. Another 10% of the total activity was eluted in the first washing. Therefore, retention of kallikrein by rabbit anti-kallikrein immunoglobulin G coupled to Sepharose 4B cannot be reproduced by the Sepharose alone, nor by coupling of non-immune-rabbit immunoglobulin G to the solid phase.

Effect of different concentrations of guanidine hydrochloride and serum albumin on the immuno-adsorption and quantitative elution of submandibular ^{125}I -labelled kallikrein

Table 1 shows the immuno-adsorption of a sample of the 100 000 g supernatant from rat submandibular glands and the quantitative recovery after elution with 4 M- and 8 M-guanidine hydrochloride. The amount of rat submandibular kallikrein that was mixed with the immunosorbent as well as the amount of enzyme eluted was determined by radioimmunoassay (Nustad *et al.*, 1979). Rat submandibular kallikrein (22–38 μg) was applied in a volume of 1 ml; no enzyme was recovered in the pooled and concentrated washings. The use of 8 M-guanidine hydrochloride did not show a consistent and substantial improvement in the quantitative recovery of the enzyme as compared with a 4 M-solution.

Table 2 shows the effect of increasing concentrations of serum albumin on the quantitative recovery of kallikrein from the immunosorbent column using 4 M-guanidine hydrochloride as eluent. An increase in the concentration of serum albumin

Table 1. Immuno-adsorption of rat submandibular kallikrein and elution by guanidine hydrochloride

The kallikrein applied was a 100 000 g supernatant and was quantified by radioimmunoassay. 0, means < 0.1 $\mu\text{g/litre}$.

Rat no.	Amount of rat submandibular kallikrein (μg)			
	Applied	Not adsorbed	Eluted with guanidine hydrochloride	
			4 M	8 M
80	32	0	6	7
80	38	0	5	9
82	22	0	2	4
82	23	0	4	4

Table 2. *Effect of albumin on immunoadsorption and elution of submandibular kallikrein by guanidine hydrochloride*
The kallikrein applied was the 100000g supernatant of rat submandibular gland. Albumin was present during the coupling of immunoglobulin G to the immunoabsorbent and in the elution buffer.

[Bovine serum albumin] (%)	Amount of rat submandibular kallikrein			
	Applied (μ g)	Not adsorbed (μ g)	Eluted with 4 M-guanidine hydrochloride (μ g)	Recovered (%)
0	81	2	19	23
0.1	132	0	44	33
0.2	236	0	75	32
0.5	201	0	132	66
1.0	201	0	133	66

Table 3. *Quantitative isolation of rat glandular kallikrein by affinity chromatography using antibody coupled to Sepharose 4B and elution with 4 M-guanidine-hydrochloride/0.5% bovine serum albumin*

Source of kallikrein	n	Amount of glandular kallikrein			
		Applied (μ g)	Not adsorbed (μ g)	Recovered	
				(μ g)	(%)
Rat pancreas	6	160, 170, 130, 44, 36, 11	2, 6, 2, 4, 4, 3	106, 82, 79, 29, 27, 7	66, 48, 61, 66, 75, 64
Rat submandibular gland	3	59, 29, 29	2, 3, 2	33, 26, 26	56, 90*, 90*
Rat lacrimal gland	2	4, 5, 5	0, 0	4, 4.5	89, 90

* Eluted twice with 4 M-guanidine hydrochloride.

up to 1% in the buffer during the immunosorption did not affect the binding between kallikrein and its antibody, which was in all situations approx. 100%. However, increasing concentrations of serum albumin raised the amount of kallikrein recovered during the elution step from 23 to 66%. Maximum recovery was obtained with 0.5% albumin, a concentration which was subsequently used when quantitative recovery was desirable.

Reproducibility of the procedure for purification of rat submandibular kallikrein by immunoadsorption

Six 1-ml samples each containing 230 μ g of rat submandibular kallikrein were subjected to immunoadsorption chromatography as described in the Experimental section. The kallikrein recovered in the washings and during elution with guanidine hydrochloride/0.5% bovine serum albumin was quantified by radioimmunoassay. The adsorption process was about 97% complete (range 97–99%) and the recovery using one elution step was about 60% (range 56–65%). When a second elution step was carried out using guanidine hydrochloride/0.5% albumin an additional 20–30% of the applied kallikrein could be recovered.

The immunosorbent column was regenerated repeatedly, and its immunological properties were preserved perfectly during a period of 6 months.

When the column was not in use, it was stored at 4°C. Antisera obtained from two different rabbits have been used with similar results.

Quantitative purification of rat glandular kallikreins by immunoadsorption chromatography

Rat pancreas and lacrimal glands were homogenized and centrifuged as described in the Experimental section. Table 3 shows the different amounts of rat glandular kallikrein applied from six pancreatic, three submandibular and two rat lacrimal gland homogenates, and the amount of the enzyme recovered from the washings and after elution with 4 M-guanidine hydrochloride/0.5% albumin. The three different rat submandibular glands were included to show the improvement in quantitative recovery when the elution step was applied twice, which increased the recovery from approx. 60% to approx. 90%. When a small amount of glandular kallikrein was applied to the immunoabsorbent column (as in the case of the lacrimal gland) a high yield was observed using only one elution step with 4 M-guanidine hydrochloride/0.5% albumin.

Electrophoretic properties of submandibular kallikrein purified by immunoadsorption chromatography

Fig. 4 shows that the eluate from the adsorption

column contains a submandibular protein preparation that moves as a single band on sodium dodecyl sulphate/16% (w/v) polyacrylamide-gel electrophoresis. Moreover, the electrophoretic mobility of

this band is similar to that of the reference kallikrein preparation.

Immunological properties of glandular kallikrein purified by immunoabsorption chromatography

Fig. 5 shows that the isolated kallikrein pre-

Fig. 4. Polyacrylamide (16%, w/v) gel electrophoresis in the presence of sodium dodecyl sulphate of submandibular kallikrein reference preparation (b) and of the preparation purified from submandibular homogenate using immunoabsorption chromatography (a)

Fig. 5. Immunoreactivity of different glandular kallikrein preparations purified by immunoabsorption chromatography

Competition of equilibrium binding to rabbit anti-(rat submandibular kallikrein) serum between submandibular ^{125}I -labelled kallikrein and glandular kallikrein preparations of different origins is shown. After immunoabsorption, the kallikrein preparations were diluted as indicated. Δ , Submandibular kallikrein; O , pancreatic kallikrein preparation; $\square$, submandibular kallikrein reference preparation. The pancreatic enzyme preparation had an immunological activity corresponding to $110\mu\text{g}/\text{mg}$ of protein.

Table 4. Specific activities of rat submandibular kallikrein after isolation by immunoabsorption

The procedures for immunoabsorption and elution of kallikrein, as well as for enzyme measurements, are described in the Experimental section. Albumin was not used in the elution buffer. The enzyme was measured at three different concentrations and the mean value and the range are given. The specific activity is different from previously reported (Brandtzaeg *et al.*, 1976) since protein was measured earlier and by using another method. The control preparation was purified according to Brandtzaeg *et al.*, 1976. Kinin-forming activity was measured in bradykinin equivalents using the rat-uterus bioassay. Bz-Arg-OEt, α -N-benzoyl-L-arginine ethyl ester; S-2266; ester substrate from Kabi Diagnostica, Stockholm, Sweden.

Kallikrein preparation	Esterase activity (units/mg of protein) with		$10^5 \times$ Kinin-forming activity ($\mu\text{g}/\text{min}$ per mg of protein)
	Bz-Arg-OEt	S-2266	
Control	338 (330–345)	456 (416–484)	1.69 (1.53–1.84)
Purified by affinity chromatography	313 (295–330)	425 (410–452)	1.62 (1.58–1.64)

parations from submandibular, pancreatic and lacrimal glands give competition curves parallel to those obtained with the reference submandibular preparation when incubated together with submandibular ^{125}I -labelled kallikreins and the antiserum. Parallel curves indicate immunological identity, even when impure preparations are used. Also, dilutions of gland homogenate gave parallel curves (data not shown). In contrast with the submandibular gland, pancreas, and possibly the lacrimal gland, contain prekallikrein. However, this antiserum reacts equally well with pancreatic prekallikrein and pancreatic kallikrein (Proud *et al.*, 1977).

Preservation of esterase and kininogenase activities of rat submandibular kallikrein after purification by immunoabsorption chromatography

Table 4 shows that the enzyme isolated by immunoabsorption chromatography has a specific activity (measured as the ability to hydrolyse α -N-benzoyl-L-arginine ethyl ester hydrochloride) comparable with that of a reference kallikrein preparation (Brandtzaeg *et al.*, 1976). Also, the specific kinin-forming activity of the purified preparation was the same as for reference submandibular kallikrein (Table 4). Therefore, the affinity chromatographic procedure gives a submandibular enzyme preparation with specific esterase and kininogenase activities very similar to those of the reference submandibular kallikrein.

Discussion

A one-step purification procedure for rat submandibular kallikrein using immunoabsorption chromatography has been described for the first time. A partly purified immunoglobulin G fraction was used and was coupled to CNBr-activated Sepharose 4B. The isolated immunoglobulin G fraction showed binding characteristic similar to those of intact antiserum against submandibular ^{125}I -labelled kallikrein. More than 97% complete adsorption was obtained for submandibular ^{125}I -labelled kallikrein and kallikreins from gland homogenate. A sharp elution profile of the adsorbed material was observed after dissociation with guanidine hydrochloride. The adsorption of ^{125}I -labelled kallikrein was biospecific, since non-activated Sepharose 4B or non-immune-rabbit immunoglobulin G coupled to Sepharose 4B was unable to bind ^{125}I -labelled kallikrein. The eluted material had the same electrophoretic mobility as a reference kallikrein preparation in conventional disc-gel electrophoresis as well as in polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, suggesting that no major physicochemical changes occurred during the isolation procedure. Moreover, the rat submandibular kallikrein isolated by immunoaffinity chromato-

graphy showed complete preservation of immunological activity, and the specific esterase and kininogenase activities were more than 90% of those of the reference preparation, indicating a satisfactory degree of purity. The quantitative recovery of eluted kallikrein was enhanced by the presence of bovine serum albumin in the buffer system. In addition, repeating the elution procedure increased the quantitative yield from approx. 60% to approx. 90%. Different concentrations of guanidine hydrochloride and the presence of bovine serum albumin in the elution buffer did not affect the absorption of kallikrein, which was always more than 97% complete. We conclude that the isolation procedure by immuno-affinity chromatography that we describe represents an efficient method for a one-step partial purification of glandular kallikreins. The specificity and completeness of the method, and the reproducibility and yield obtained, make it a very attractive procedure either for the rapid isolation of glandular kallikrein for further characterization, or to examine the presence of glandular kallikreins in various organs and biological fluids.

Financial support from the Norwegian Cancer Society is gratefully acknowledged. We thank engineer K. H. N. Lyngaas for help with the bioassay.

References

- Brandtzaeg, P., Gautvik, K. M., Nustad, K. & Pierce, J. V. (1976) *Br. J. Pharmacol.* **56**, 155–167
- Gallimore, M. J., Aasen, A. O., Lyngaas, K. H. N., Larsbraaten, M. & Amundsen, E. (1978) *Thromb. Res.* **12**, 307–318
- Gautvik, K. M., Berg-Ørstavik, T., & Nustad, K. (1976a) in *Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease* (Pisano, J. J. & Austen, K. F., ed.), pp. 335–357, Fogarty International Center Proceedings no. 27, U.S. Government Printing Office, Washington DC
- Gautvik, K. M., Kriz, M. & Fossum, S. (1976b) *Anal. Biochem.* **73**, 52–62
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nustad, K. & Pierce, J. V. (1974) *Biochemistry* **13**, 2312–2319
- Nustad, K., Pierce, J. V. & Vaaje, K. (1975) *Br. J. Pharmacol.* **53**, 229–231
- Nustad, K., Ørstavik, T. B., Gautvik, K. M. & Pierce, J. V. (1978) *Gen. Pharmacol.* **9**, 1–9
- Nustad, K., Gautvik, K. M. & Ørstavik, T. (1979) in *Kinins II: Biochemistry, Pathophysiology and Clinical Aspects* (Fujii, S., Moriya, H. & Suzuki, K., eds.), pp. 225–233, Plenum Publishing Corp., New York
- Ørstavik, T. B., Gautvik, K. M. & Nustad, K. (1980) *Acta Physiol. Scand. (Copenhagen)*, in the press
- Proud, D., Bailey, G. S., Nustad, K. & Gautvik, K. M. (1977) *Biochem. J.* **167**, 835–838