The major androgen-dependent protease in dog prostate belongs to the kallikrein family: confirmation by partial amino acid sequencing

C. Lazure*, R. Leduc*, N.G. Seidah*, M. Chrétien*, J.Y. Dubé+, P. Chapdelaine+, G. Frenette+, R. Paquin+ and R.R. Tremblay+

Laboratories of Biochemical and Molecular Neuroendocrinology Clinical Research Institute of Montréal, 110 Pine Avenue West, Montréal, Canada H2W 1R7 and *+Laboratory of Endocrinology and Metabolism Laval University Hospital Centre Ste-Foy, Canada G1V 4G2*

Received 23 July 1984

Canine prostate fluids and seminal plasma contain a major androgen-dependent protein which was identified as a proteolytic enzyme exhibiting an Arg-esterase activity. This protease, as characterized, is shown to be present as a two-chain structure held together by at least one disulfide bridge and composed of approximately 220 amino acids. Amino acid sequence determination of both chains has revealed a clear homology to other known amino acid sequences of serine proteases. Furthermore, the comparison of the presented 58 amino acids of the Arg-esterase with the other sequences revealed a very strong homology (larger than 50%) to members of the kallikrein family. The two chain structure could thus result from autolysis of a single chain enzyme in the 'kallikrein autolysis loop'. Amino acid composition of the canine prostatic enzyme suggests that it is related, but not identical, to pancreatic canine kallikrein.

Androgen-dependent protease *Canine prostate* *Kallikrein autolysis loop*

1. INTRODUCTION

Potent proteolytic enzymes, at first detected by their fibrinolytic and fibrinogenolytic properties, have long been known to exist in seminal plasma [1]. Indeed, some early reports [2,3] have described the presence in canine prostatic fluids of numerous proteolytic activities including some akin to an Arg-esterase activity. Similarly, a kallikrein-like enzyme or kininogenase, as evidenced by its ability to generate kallidin from crude dog kininogen and to induce a significant vasodilator effect in dog, was shown to be present in the accessory sex glands and in the prostate gland of the guinea pig [4]. However, such a kinin-producing activity was not observed in the case of the enzymes present in the coagulating gland of the rat or in the prostate gland of rat, rabbit, dog and man [4]. Recently, one of the major protein constituents of canine seminal fluid was found to be rapidly synthesized when canine prostatic slices were incubated with radioactive amino acids; furthermore, both the amount synthesized and the kinetics were greatly affected by the level of testicular hormones as evidenced in castrated animals and upon injection of androgens [5]. Furthermore, this major androgen-dependent protein in canine prostate was shown to be an Arg-esterase that was inhibited upon addition of diisopropyl fluorophosphate, a potent inhibitor of serine proteases [6].

The present paper validates the identification of that major androgen-dependent protease as being a serine protease and, moreover, identifies it unambiguously as belonging to the kallikrein fami-

* *Correspondence should be sent to:* Dr Michel Chrétien, 110 Pine Avenue West, Montréal, Canada H2W 1R7

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

ly. Furthermore, this Arg-esterase, in the form isolated, possesses a two-chain structure resulting from autolysis in a manner analogous to β-kallikrein.

2. MATERIALS AND METHODS

Native arginine esterase was isolated by chromatofocusing [6]. Only the first peak eluting at pH 7.3 was used for sequence studies. This fraction was found to contain only two protein spots in apparently equal amounts in two-dimensional non-equilibrium pH gel electrophoresis [6]. It was dialyzed extensively against distilled water and was lyophilized. The heavy fragment arising from autolysis was isolated by a procedure involving a desalting step on Sephadex G-25 followed by chromatography on CM-Sephadex to remove high molecular weight acidic proteins. After desalting and lyophilization, the material was subjected to non-equilibrium pH gel electrophoresis in tubes as described in [7]. The most basic protein was recovered from the gel by electroelution [8]. It was desalted over Sephadex G-25 and dialyzed extensively against distilled water to remove contaminating ampholytes. After lyophilisation, the recovered material was found to contain a single spot by two-dimensional gel electrophoresis.

The disulfide bridges of the native Arg-esterase were opened by reduction with dithiothreitol and the resulting half-cystines were alkylated with iodoacetic acid as proposed in [9]. The protein was desalted on a Sephadex G25 column eluted with ammonium bicarbonate 1% and recovered by lyophilization. Native, reduced and alkylated Arg-esterase were subsequently analyzed by high-performance liquid chromatography (HPLC) on a large pore (330 Å) VYDAC C_{18} column (0.46×25 cm) using 0.1% trifluoroacetic acid as eluant and 1-propanol as organic modifier. The HPLC apparatus consisted of a Waters model 204 liquid chromatograph equipped with a model 730 integrator-plotter and a model 720 system controller.

Amino acid analyses were done following acid hydrolysis in 5.7 N HCl containing a trace of phenol and 0.01% mercaptoethanol in vacuo at 108°C. The separation and quantitation of the amino acids were performed on a Beckman 121 M auto analyzer.

Automatic amino-terminal Edman degradation on the native material was performed using a 0.33 M Quadrol program on a Beckman 890 C sequenator equipped with a Sequemat P6 autoconverter and a model SC-510 controller. Moreover, 3 mg of Polybrene (Aldrich) and addition of *o*-phthalaldehyde (OPA) (Fluoropa, Pierce Chem) at selected cycles suspected to be proline residues were both used in [10]. PTH-amino acid derivatives were identified and quantitated using PTH-norleucine as internal standard by HPLC [11], on a Varian 5560 liquid chromatograph equipped with a Vista 402 integrator-plotter and with a WISP (Waters) automatic injector.

3. RESULTS

Preliminary results obtained while sequencing the native enzyme revealed the presence of two amino terminal sequences in almost equal proportion, one of which possessed a Pro residue at cycle 13. Therefore, the native enzyme was sequenced again but, this time, addition of OPA at cycle 13 allowed the identification of a single sequence as shown in fig. 1A. The initial and repetitive yields were computed after background correction to be 4.7 nm and 92.7%, respectively. Not shown on the figure is the sequence of the first 12 cycles corresponding to Ala-Val-Ile-Arg-Pro-Gly-Glu-Asp-Arg-Ser-His-Asp and representing the second sequence. As can be seen in fig. 1A, no amino acid derivatives were identified at cycle 7 and 26; both of these positions are presumed to be occupied by half-cystine. Amino acid sequence analysis of the basic Arg-esterase fragment, isolated following NEpHGE, is shown in fig. 1B. It can be clearly seen that the first 12 residues identified correspond entirely with the amino acids identified while sequencing the native enzyme. The initial and repetitive yields for that sequence were computed after background subtraction to be 9.4 nm and 94% respectively. Both sequences were not pursued further because of an increasing background probably caused by the methods used during isolation.

To further conform the presence of a two-chain structure for that protease as suggested by the results of the amino acid sequence analysis, the native enzyme was purified by reverse-phase HPLC as shown in fig. 2A where it can be eluted

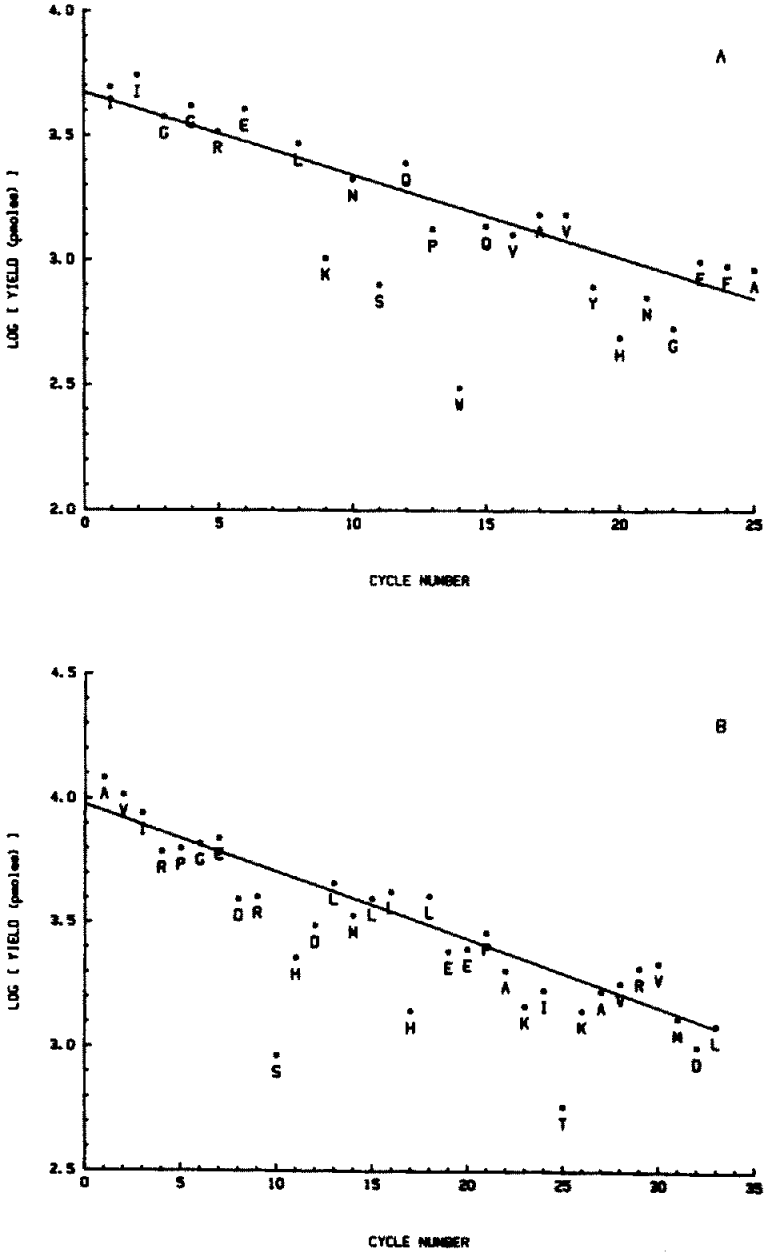

Fig. 1. Automatic NH_2-terminal degradation of native canine Arg-esterase (A) and of a fragment arising from autolysis (B). Quantitative yields after background subtraction of PTH-amino acids normalized to a PTH-norleucine internal standard are illustrated as a function of residue numbers. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids giving the repetitive and initial yield, respectively.

as a single symmetrical peak at around 24 min. Amino acid compositions of that material were done and the results are presented in table 1. Upon reduction and alkylation of the HPLC purified enzyme, two homogeneous components, as determined by sequence analysis (results not shown) eluting at 23 and 25 min, respectively, can be separated, as illustrated in fig. 2B. Amino acid compositions of the material under each peak were done and the results presented in table 1. As can be seen, the Arg-protease is composed of a light chain containing 3 half-cystine residues corresponding to the early eluting component and of an heavy chain containing 7 half-cystine residues corresponding to the later eluting peak. That result was independently confirmed by labeling the half-cystine with radioactive iodoacetic acid and separation of the two components by HPLC; the total recovery of radioactivity was found to be 86.3%, whereas the radioactivity content corresponded to 34 and 66% for the early and later eluting peak, respectively (results not shown). Moreover, it can be seen that if one makes the summation of the amino acid content of each peak and compares the results with the total amino acid composition of the native enzyme, they are in very close agreement. The resulting amino acid composition thus suggests that the Arg-esterase, as isolated from canine seminal fluids, should contain ca. 220 amino acids.

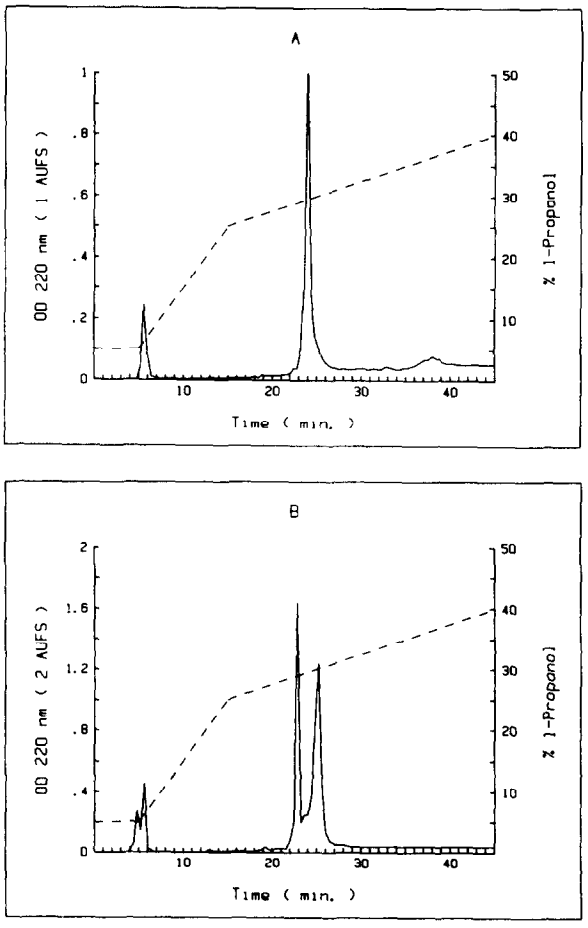

Fig. 2. HPLC chromatography of the native (A) and the reduced and alkylated (B) canine Arg-esterase on a Vydac C_{18} column eluted with a 0.1% TFA-1-propanol gradient at a flow rate of 1 ml/min.

Table 1

Amino acid composition of canine prostate ARG-esterase

Amino acids	Light chain[a]	Heavy chain[b]	Native	Canine kallikrein[c] (pancreas)
Asx	6.3 (6)	10.4 (10)	17.3 (17)	25
Thr	1.9 (2)	8.8 (9)	13.9 (14)	15
Ser	4.9 (5)	7.5 (8)	14.1 (14)	14
Glx	12.4 (12)	11.9 (12)	22.7 (23)	26
Pro	3.3 (3)	9.5 (9–10)	13.5 (13–14)	15
Gly	8.6 (9)	13.4 (13)	19.6 (20)	20
Ala	5.1 (5)	6.3 (6)	11.2 (11)	15
Val	10.3 (10)	11.6 (12)	20.5 (20–21)	17
Met	-	4.5 (4–5)	5.2 (5)	4
Ile	1.8 (2)	4.5 (4–5)	7.3 (7)	12
Leu	6.9 (7)	14.0 (14)	22.7 (23)	23
Tyr	2.7 (3)	3.1 (3)	4.8 (5)	8
Phe	2.4 (2)	2.8 (3)	3.9 (4)	8
His	4.3 (4)	4.0 (4)	8.0 (8)	8
Lys	3.8 (4)	12.3 (12)	16.2 (16)	13
Arg	3.6 (4)	3.7 (4)	7.2 (7)	5
CM-Cys	3.0 (3)	7.0 (7)	-	8
Trp	ND[d]	ND[d]	ND[d]	4
Total	81	134–137	207–209	240

[a]Results obtained from 24 h hydrolysis
[b]Results obtained from duplicate hydrolysis during 24, 48 and 72 h
[c]Values taken from [20]
[d]ND, Not determined.
The compositions were adjusted according to the underlined residues.

4. DISCUSSION

Following the study concerning the effect of testicular hormones on synthesis of soluble proteins by dog prostate slices, it was proposed that the major androgen-dependent protein seen as a 15 kDa protein could be part of a serine-protease considering its ability to bind DFP [6]. This peptide was later shown to be part of a bigger protein exhibiting a molecular mass of 29.5 kDa as estimated by gel filtration and able to hydrolyze BAEE, a known Arg-esterase substrate. Upon reduction of the disulfide bridges, two chains corresponding to 15 kDa and 14 kDa, respectively, were released, the former being able to bind DFP while the latter, in view of its electrophoretic heterogeneity, is probably glycosylated [6].

As can be seen in fig. 3, the partial amino acid sequence of both chains can be compared with the amino acid sequences of kallikrein-related proteases. The homologies thus range from 49% with the nerve growth factor γ-subunit [15] up to 64.4% with the porcine pancreatic kallikrein [14], whereas it is only 28% with bovine trypsin and chymotryp-

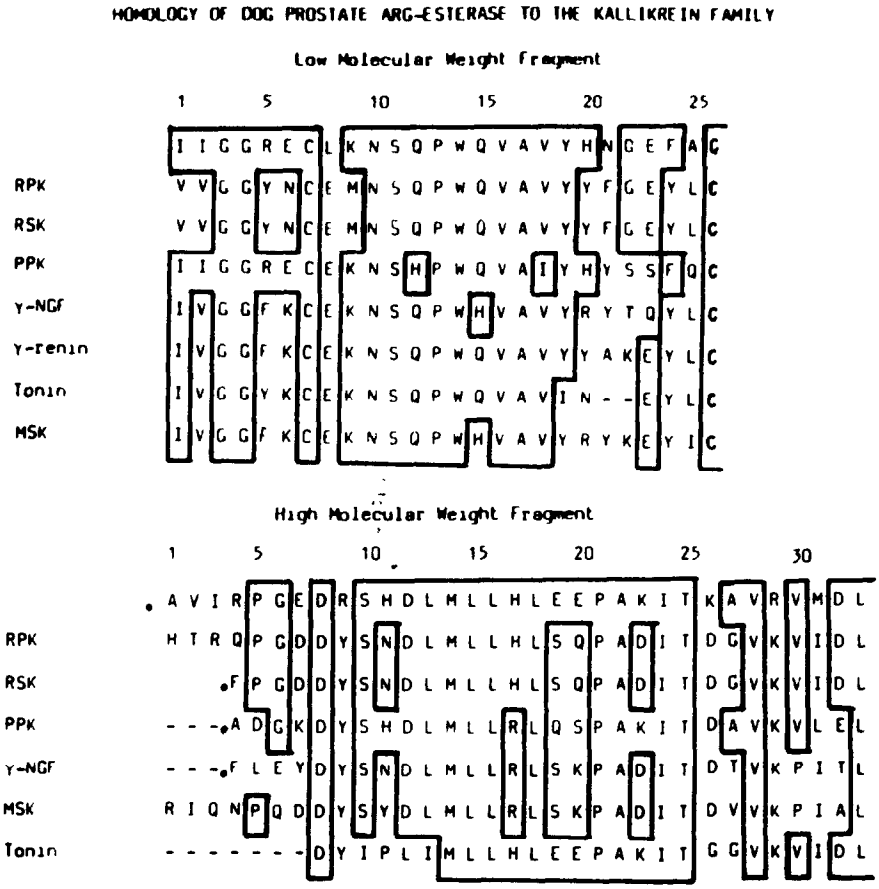

Fig. 3. Comparison of the NH_2-terminal sequence of the two fragments obtained from canine prostate Arg-esterase (top lines) with corresponding sequence aligned by homology of rat pancreatic kallikrein (RPK) [12], rat submaxillary gland kallikrein (RSK) [13], pig pancreatic kallikrein (PPK) [14], γ-subunit of NGF [15], γ-renin [16], rat tonin [17] and mouse submaxillary gland kallikrein (MSK) [18]. Identical residues are enclosed in boxes and the sign • in front of a sequence indicates the beginning of an observed autolytic fragment.

sin. Moreover, the alignment of both sequences confirms the nature of both chains and also confirms the results in [5,6]. Indeed, the early eluting peak in HPLC corresponds to the lower molecular weight chain and to the NH_2-terminal sequence starting at Ile-Ile-Gly... Therefore, based upon the amino acid composition, it should extend up to 80–85 residues; the Asn-78 found in the porcine pancreatic kallikrein and known to be linked to carbohydrates could be present and could explain the observed electrophoretic heterogeneity. Also, the presence of the presumed half-cystine at position 7 is responsible for the linkage to the other chain as can be surmised from the homology to other kallikreins. The higher molecular weight chain corresponding to the later eluting peak should comprise the remainder of the molecule (ca. 137 residues). When compared with other kallikreins, it is clearly produced by an autolytic cleavage in the known 'kallikrein autolysis loop' since the beginning of its sequence is very close or other observed autolytic sites found in rat submaxillary [13] and porcine pancreatic [14] kallikrein or the γ-subunit of NGF [15]. Moreover, it contains the active site serine (responsible for binding DFP) and Asp residues (found at position 12 of the high molecular weight fragment in fig. 3). On the other hand, it cannot be said, as yet, whether that structure of ca. 220 amino acids fully represents the complete canine Arg-esterase since some amino acids in between the two chains could have been lost during the autolytic process.

Further comparisons can also be made between the amino acid composition and the structural characteristics of the canine Arg-esterase and of a glycoprotein also present in canine prostatic fluid [19]; indeed, when one recalculates the amino acid composition of the latter according to the data reported herein, it can be suggested that both proteins are identical even though, in the latter case, no enzymatic activities were studied. Moreover, the amino acid composition of the canine Arg-esterase seems to be significantly different from the one reported in [20] for the canine pancreatic kallikrein shown in the last column of table I, even when considering the possible losses of some amino acids due to autolysis between the two chains. Considering that the murine genome encodes numerous highly homologous genes for kallikrein [18], it could thus mean that two different structural genes are expressed in both canine tissues, the pancreas and the prostate gland. On the other hand, the validity of such an affirmation cannot be clearly assessed at the moment. Indeed comparison of immunological data and amino acid compositions of porcine pancreatic, submaxillary gland and urinary kallikrein, as reviewed in [21], and also comparison of the amino acid sequence of rat submaxillary gland [13] and rat pancreatic [12] kallikrein where 117 positions out of 118 are identical seems to indicate that the same structural gene is expressed in different tissues.

Since it is not yet known whether the canine enzyme is able to release kinins (preliminary data [4,5] seem to indicate that it cannot), its similarity to the γ-subunit together with the high amount present in the prostate gland are intriguing. The prostate gland of guinea pig, rabbit and bull were shown to contain substantial amount of NGF [22] but it is not known whether NGF is present in substantial amount in the prostate gland of the dog. Further data is thus needed either on the presence of NGF or upon the nature of the canine γ-subunit before any conclusions can be made on the possible coexistence of NGF and the canine Arg-esterase. On the other hand, the presence of a kininogenase in human seminal plasma able to release kinins from kininogen was reported [23,24] whereas previous reports had failed to show production of kinins; the same could hold true for the canine Arg-esterase.

Finally, because of its sensitivity to androgen modulation and its now established relationship to kallikreins, ' group of serine proteases with bioregulatory actions', the canine Arg-esterase present in seminal fluids should provide an interesting marker for the study of benign and malignant neoplasia and of the mechanism of steroid hormone action, the dog prostate being a model well suited for both aspects. Further chemical characterization of the canine Arg-esterase is currently in progress in our laboratories.

ACKNOWLEDGEMENTS

We are grateful to Ms D. Marcil for the help in preparing the manuscript. This work was supported by a Program Grant (PG-2) from the Medical Research Council of Canada. C.L. is a 'chercheur-boursier' from the F.R.S.Q.

REFERENCES

[1] Mann, T. (1954) in: The Biochemistry of Semen, pp. 1–194, Methuen, London.

[2] Gotterer, G., Banks, J. and Williams-Ashman, H.G. (1956) Proc. Soc. Exp. Biol. Med. 92, 58–61.

[3] Rosenkrantz, H. and Kirdani, E.S. (1961) Cancer Chem. Rep. 15, 9–16.

[4] Bhoola, K.D., Yi, R.M.M., Morley, J. and Schacter, M. (1962) J. Physiol. 163, 269–280.

[5] Dubé, J.Y., Chapdelaine, P. and Tremblay, R.R. (1983) Can. J. Biochem. Cell. Biol. 61, 756–763.

[6] Chapdelaine, P., Dubé, J.Y., Frenette, G. and Tremblay, R.R. (1984) J. Androl. 5, 206–210.

[7] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell 12, 1133–1142.

[8] Otto, M. and Snejdarkova, M. (1981) Anal. Biochem. 111, 111–114.

[9] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.

[10] Lazure, C., Seidah, N.G., Chrétien, M., Thibault, G., Garcia, R., Cantin, M. and Genest, J. (1984) FEBS Lett. 172, 80–86.

[11] Lazure, C., Seidah, N.G., Chrétien, M., Lallier, R. and St.-Pierre, S. (1983) Can. J. Biochem. Cell. Biol. 61, 287–292.

[12] Swift, G.H., Dagorn, J.O., Ashley, P.L., Cummings, S.W. and MacDonald, R.J. (1982) Proc. Natl. Acad. Sci. USA 79. 7263–7267.

[13] Lazure, C., Seidah, N.G., Thibault, G., Genest, J. and Chrétien, M. (1981) in: Proceedings of the 7th American Peptide Symposium (Rich, D.H. and Gross, E., eds) pp. 517–519, Pierce, New York.

[14] Tschesche, H., Mair, G., Godec, G., Fiedler, F., Ehret, W., Hirschauer, C., Leman, M. and Fritz, H. (1979) Adv. Exp. Med. Biol. 120A, 245–260.

[15] Thomas, K.A., Baglan, N.C. and Bradshaw, R.A. (1981) J. Biol. Chem. 256, 9156–9166.

[16] Poe, M., Wu, J.K., Florance, J.R., Rodkey, J.A., Bennett, C.D. and Hoogsteen, K. (1983) J. Biol. Chem. 258, 2209–2216.

[17] Lazure, C., Leduc, R., Seidah, N.G., Thibault, G., Genest, J. and Chrétien, M. (1984) Nature 307, 555–558.

[18] Mason, A.J., Evans, B.A., Cox, D.R., Shine, J. and Richards, R.I. (1983) Nature 303, 300–307.

[19] Isaacs, W.B. and Shaper, J.H. (1983) J. Biol. Chem. 258, 6610–6615.

[20] Hojima, Y., Yamashita, M., Ochi, N., Moriwaki, C. and Moriya, H. (1977) J. Biochem. (Tokyo) 81, 599–610.

[21] Fiedler, F. (1979) In: Handbook of Experimental Pharmacology (Erdos, E.G., ed) vol. XXV, pp. 103–161, Springer-Verlag, Berlin.

[22] Harper, G.P., Barde, Y.A., Burnstock, G., Carstaus, J.R., Dennison, M.E., Suda, K. and Vernon, C.A. (1979) Nature 279, 160–162.

[23] Geiger, R. and Glausnitzer, B. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1279–1283.

[24] Fink, E. (1984) In: Protides of the Biological Fluids, Vol. XXXII, in press.